(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 March 2008 (20.03.2008)

PCT

(10) International Publication Number
WO 2008/032314 A2

(51) International Patent Classification:
A01N 1/02 (2006.01)

(21) International Application Number:
PCT/IL2007/001120

(22) International Filing Date:
11 September 2007 (11.09.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/843,394 11 September 2006 (11.09.2006) US

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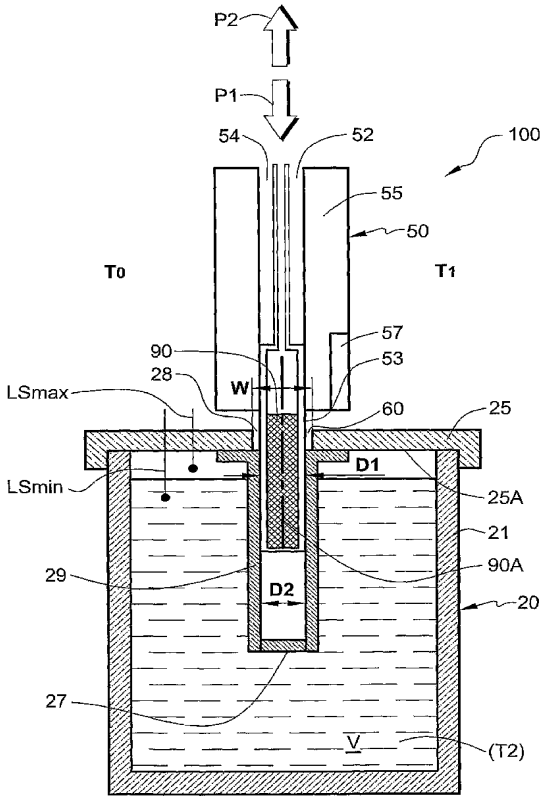
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH,

[Continued on next page]

(54) Title: SYSTEMS, DEVICES AND METHODS FOR FREEZING AND THAWING BIOLOGICAL MATERIALS



(57) Abstract: There are provided a system and a method for cryopreserving a liquid biological material disposed in a bag having a longitudinal axis. The system comprises a bag holder for holding the bag, so that the biological material therein has a surface area S, and a volume V, a tank containing a cryogenic fluid, a mechanism for the immersion of the bag holder into the tank along the longitudinal axis, an opening in the tank for insertion therethrough of the bag holder, and a guide member extending from the opening into the tank. There are further provided a system and a method for warming a cryopreserved liquid biological material disposed in a bag. The system comprises a heat source, a warming device having a space for placing the bag therein, connected to the heat source and adapted to transfer heat from the heat source to the bag, and means for maintaining the heat source in heat transfer contact with a cryogenically preserved portion of the material to allow receiving the heat by said cryogenically preserved portion.

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GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

SYSTEMS, DEVICES AND METHODS FOR FREEZING AND THAWING BIOLOGICAL MATERIALS

FIELD OF THE INVENTION

The present invention relates to apparatuses and methods for freezing and thawing liquid biological materials; e.g. blood and blood components.

BACKGROUND

Cryopreservation of biological material is intended to extend the preservation period of biological material while maintaining their functionality and viability after warming. This may be done by freezing or vitrification. In vitrification, a vitrification solution is added to the biological material, such that the freezing point of the sample is reduced, which in turn leads to vitrification rather than freezing (i.e. ice is essentially not formed). Freezing is a transient non-equilibrium process, during which ice crystallization occurs with release of latent heat as liquid or fluid cools below freezing temperature due to ambient cooling conditions.

Different biological materials require different cooling rates in order to survive cryopreservation. At times the best cooling rates are low (e.g. 0.5-5°C/min) while for other biological materials the best survival is observed at relatively high cooling rates (>5°C; e.g. ca. 50°C/min for semen and 500-5,000 °C/min for red blood cells).

The cooling rate during cryopreservation is affected by factors that include the volume of the biological material, its geometrical shape, and the cooling method (i.e. liquid nitrogen, nitrogen vapor, freezers, dry ice, etc.). When the samples are bulky, the rate of removal of heat through the bulk of the sample may limit the cooling rate.

In directional cooling (such as disclosed in US 5,873,254, entitled "Device and method for multigradient directional cooling and warming of biological samples") biological material is frozen by being moved along a temperature gradient. In such cases, the cooling rate is also influenced by the velocity of movement along the gradient.

The following references are presented by way of general background interest.

US 5,863,715, entitled "Methods for bulk cryopreservation encapsulated islets" discloses method for cryopreservation of encapsulated islets. The method includes the steps of providing a flexible container, such as a freezer bag, containing the islets; placing the bag in a holder that maintains the cross-sectional area of the bag essentially constant and small; treating it with a cryoprotectant and freezing it.

US 4,018,911, entitled "Method for large volume freezing and thawing of packed erythrocytes" discloses a rigid metal holder having perforated side plates for containing plastic bags of human red blood cells for both freezing and thawing such cells is provided. Hydroxyethylstarch, HES, is used as the cryoprotective agent.

US 5,309,723, entitled "Method of freezing red blood cells", discloses a method of freezing a standard donor unit of red blood cells and involves centrifuging a blood unit to remove plasma and platelets and to provide a Packed Cell Volume of the red blood cells of not less than 90%, adding the red blood cells to a freezing bag, containing HES solution such that the ratio of HES/red blood cell freezing unit is not more than 7% (preferably 6%) w/v, positioning the freezing bag in a freezing frame adapted to maintain the thickness of the contents of the bag constant, and placing the frame without shaking, into liquid nitrogen.

US 5,935,848, entitled "Deep-freezing container" discloses a container having two plane-parallel thin-walled metallic plates secured in swivellingly interconnected frame halves. When the frame halves are superimposed, the plates secured therein are arranged with parallel faces at a slight defined distance from each other and form an intermediate cavity in which the bag is placed. When the container is closed, the plates press the bag arranged inside the container and flatten it until it has a small defined thickness. A microporous layer is secured to the outer side of plates by an adhesive layer.

US 6,022,344, entitled "Cryopreservation bag", relates to a bag for the cryopreservation of blood cells, comprising a joining piece and a shrink tube to connect the bag with a non-PVC tubing.

US 4,327,799, entitled "Process and apparatus for freezing living cells" discloses a process for freezing cell suspensions by locating the suspension in a freezing chamber and simultaneously monitoring the temperature of the suspended cells and of the chamber. The cooling of the chamber is regulated at predetermined rates in response to

give temperature levels of the sample. The cooling chamber includes a fan, a heater, and a source of refrigerant. The process includes the steps of selectively decreasing and increasing the temperature of the freezing chamber responsive to predetermined temperature points on the freezing curve of the cell sample.

US 5,250,044, entitled "Blood cryopreservation container", relates to bags for the cryopreservation of mammalian cells and particularly for the long-term freezing of red blood cell, and to methods of manufacturing such bags.

US 2005/129887, entitled "Medical freezer bag", discloses a medical freezer bag made from a three-layer film where both sides of an ultra-high molecular weight polyethylene (PE) film are welded respectively with a thermoplastic resin film having a lower melting point than that of the ultra-high molecular weight polyethylene and having compatibility with the ultra-high molecular weight polyethylene.

WO 2004/003444, entitled "Changing the temperature of a liquid sample and a receptacle useful therefor", discloses a method of changing the temperature of a liquid sample, comprising: providing a receptacle having inner and outer walls defining an annular portion therebetween for receiving therein a liquid sample, inserting said liquid sample, at a first temperature, into said annular portion, and exposing said receptacle to a second temperature different from said first temperature. Further disclosed are a receptacle useful in the method and a chamber useful for performing said method.

WO 2005/072523, entitled "Biological material and methods and solutions for preservation thereof", discloses a preservation solution for preserving biological material at low temperature comprising one or more polyphenols and a method for preservation of biological material, said method comprising adding the preservation solution to biological material, cooling the biological material and storing it at appropriate storing conditions. The method may be used for hypothermic preservation or for cryopreservation, including freezing and lyophilization, and may be used with any biological material, including cells selected from RBC, WBC, MNC, UCB MNC and bacteria. In case of RBC the present invention also disclosed a method for its freezing such that upon thawing, the material has less than 2% free hemoglobin.

SUMMARY OF THE INVENTION

Herein, "biological material" includes any material which comprises biological material (e.g., cells, tissue, or portion thereof), and may include, inter alia, any liquid

suspension comprising biological cells, blood, blood components, blood fractions, blood preparations, blood plasma, semen, stem cells, bone marrow cells, platelets, oocytes, embryos, lymphocytes, white blood cells and/or red blood cells. At times, the biological material is biological material havning a preferred freezing rate of above 1°C/min, at times above 5°C or even 100°C/min or more.

The term "cryopreservation" means vitrification or freezing, and "cryopreserved biological material" means vitrified and/or frozen biological material in an essentially solid state. The term "warming" of a cryopreserved biological material means the warming of such material, which may be performed until the major portion or whole of the biological material becomes liquid. In case of frozen biological material the warming may mean thawing, while in the case of vitrified biological material, warming may mean liquefying.

According to one aspect of the present invention, there is provided a system for cryopreserving a liquid biological material disposed in a bag having a longitudinal axis, the system comprising:

- a bag holder for holding said bag, so that the biological material therein has a surface area S, and a volume V;
- a tank containing a cryogenic fluid, such as liquid nitrogen or the like;
- a mechanism for the immersion of said bag holder into said tank along said longitudinal axis;
- an opening in said tank for insertion therethrough of the bag holder; and
- a guide member extending from said opening into said tank and adapted for engaging walls of said bag holder, when the bag holder is being immersed into the tank via said opening, to reduce widening of the bag during its being frozen by the cryogenic fluid.

The tank and the immersion mechanism constitute together a submersion freezing device, also referred as SF device or SFD.

The bag preferably comprises a high surface area-to-volume ratio, and a substantially constant width (e.g. about 4mm to about 27mm, in some embodiments preferably no more than 15mm), which helps to provide uniformity of thermal history through the biological material (or a major portion thereof) where appropriate, and/or to reduce the likelihood of the bag being damaged during freezing, thawing, handling,

storage, transportation, and so on, and/or to potentially increase the post-thaw viability of at least some types of biological materials, for example blood cells or other cells.

Any one or more of the following features may be included in the system:

- The guide member may comprise a plurality of guides in the form of tongues depending into said tank from said opening and disposed on two sides of the longitudinal axis of the bag, which define therebetween a narrowing path and may also provide increasing lateral pressure on the bag before freezing thereof.
- The guides may be spaced by a proximal distance at a proximal portion of the guide member and a distal distance at a distal portion of the guide member, said distal distance being equal to or lesser than said proximal distance.
- The tank may comprise a cover, wherein the proximal portion of the guide member is attached to said cover at the opening's periphery. The cover may have an inner surface facing the interior of the tank, and the proximal portion of the guide member is attached to the inner surface.
- The tank may comprise level sensors for monitoring a level of the cryogenic fluid within the tank.
- The tank may comprise a floating buoy for preventing overflowing of the cryogenic fluid within the tank.
- The bag holder may be in the form of a cartridge comprising a pair of frame members, which constitute side walls of the cartridge, for placing the bag with the biological material in a space created therebetween. The cartridge along or in combination with a holding structure in the tank reduces or prevents forming irregularities or bulges in the shape of the bag.
- The bag holder may further comprise stiffening members to ensure that the bag maintain a generally uniform width when filled with biological material and is held vertically in the cartridge and during freezing.
- The system may comprise means for keeping volume of said cryogenic fluid in said tank within a certain range during the immersion of the bag with the biological material. The main purpose is to keep the cryogenic fluid surface at a certain level (within tolerance of 2-3 cm), which may be obtained by

automatic control with level sensors or floating sensor and electromagnetic cryogenic valve.

- The system may further comprise a temperature control block (such as a heated block of thermo-conductive metal) for setting an immersion temperature of said bag to a pre-determined value higher than a temperature of said cryogenic fluid, thus providing a sharp temperature gradient in a relatively short distance, which may play a role in providing controlled directional freezing and may help to orient the ice crystals formed during the freezing process in a desired upward and inward direction. The temperature control block may be adapted for setting said immersion temperature to between about 0°C and about 30°C. In some embodiments the temperature is maintained at above room temperature, in which case cooling of the control block may be unnecessary. The temperature control block provides a starting temperature which is essentially independent of (or above) the ambient temperature. Since the starting and freezing conditions are controlled, this feature provides greater repeatability in terms of the freezing process than may be obtained where the starting conditions are not controlled and are therefore subject to fluctuation. Similarly, better repeatability may also be provided in terms of the post thaw viability results.
- The system may further comprise a velocity control means allowing to select a pre-determined value of said immersion velocity and to maintain said pre-determined value constant during said immersion. The immersion velocity can be chosen optimally to maximize and reduce or prevent variations in the heat transfer between the bag and the cold source (while reducing or preventing boiling of the liquid nitrogen, when this is the cold source, which may happen when the immersion velocity is too fast), while reducing the exothermic reaction period associated with the freezing and/or increasing the cooling rate of the biological material. It is noted that a high velocity of immersion may cause boiling of the cryogenic fluid which would significantly reduce the temperature gradient between the fluid and the bag during freezing, thereby reducing the cooling rate at least in some portions of the biological material. Alternatively, the immersion velocity can be chosen

optimally to maximize and prevent variations in the post thaw viability of the biological material.

- The system may further comprise means for reducing boiling of the cold temperature source. Such means may include, without limitation, suitable cooling means for cooling the cold source to as low temperature as possible without solidification (e.g. liquid nitrogen may be cooled down to about -210°C, but above -214°C, without solidification).
- The system may further comprise means for increasing the volume of the cold temperature source and/or adding flow or turbulence thereto. Further, the cold temperature source may be chosen to provide a desired cold temperature, being below the freezing temperature of the biological material, and optionally below -80°C, below -100°C or even lower. Examples of cold temperature sources may include liquid nitrogen, liquid helium, liquid hydrogen, and liquid alcohols.

According to another aspect of the present invention, there is provided a system for cryopreserving a liquid biological material disposed in a bag having a longitudinal axis, the system comprising:

- a bag holder for holding said bag, so that the biological material therein has a surface area S, and a volume V;
- a tank containing a cryogenic fluid;
- a mechanism for the immersion of said bag holder into said tank along said longitudinal axis at an immersion velocity, wherein said immersion velocity may be of a plurality of values; and
- a velocity control means allowing to select a pre-determined value of said immersion velocity from said plurality of values, and to maintain said pre-determined value constant during said immersion.

According to another aspect of the present invention, there is provided a system for cryopreserving a liquid biological material disposed in a bag having a longitudinal axis, the system comprising:

- a bag holder for holding said bag, so that the biological material therein has a surface area S, and a volume V;
- a tank containing a cryogenic fluid;

- a mechanism for the immersion of said bag holder into said tank along said longitudinal axis; and
- a temperature control block for setting an immersion temperature of said bag, prior to said immersion, to a pre-determined value higher than a temperature of said cryogenic fluid.

According to another aspect of the present invention, there is provided a system for cryopreserving a liquid biological material disposed in a bag having a longitudinal axis, the system comprising:

- a bag holder for holding said bag so that the biological material therein has a surface area S, and a volume V;
- a tank containing a cryogenic fluid;
- a mechanism for the immersion of said bag holder into said tank along said longitudinal axis; and
- means to prevent ratio between the of the surface area S and that of the volume V of said biological material from increasing during the immersion to more than 4:3.

The bag when held by the bag holder has two large side surfaces and a thickness therebetween which is significantly smaller than the dimensions of the large side surfaces, wherein said surface area is the total area of the side surfaces. The prevention of, the surface area-to-volume ratio of the biological material from increasing may be obtained by reducing, or even eliminating, the widening, i.e. increasing the thickness, of the bag during the immersion. This may be achieved, for example, by using the guides, selecting the rate of the immersion or using the stiffening members, or by a combination of any of them.

According to another aspect of the present invention, there is provided a method for cryopreserving a liquid biological material disposed in a bag having a longitudinal axis, the method comprising:

- placing the bag in a bag holder; and
- immersing said bag holder into a cryogenic fluid in a direction parallel to said longitudinal axis at an invariant immersion velocity selected so as to ensure that the thermal curve of the biological material at least at three different locations along the longitudinal axis of the bag is essentially as shown in Fig. 11.

According to another aspect of the present invention, there is provided a method for cryopreserving a liquid biological material disposed in a bag having a longitudinal axis, the method comprising:

- placing the bag in a bag holder; and
- immersing said bag holder into a cryogenic fluid in a direction parallel to said longitudinal axis at an invariant immersion velocity selected so as to ensure that cooling behavior of the biological material in a range of temperatures T within time t at least at three different locations along the longitudinal axis of the bag is defined by a T(t) curve having initial curved portion, central essentially linear portion and final curved portion, the central portions corresponding to more than half of the range of temperatures, and at least the central portions being parallel at said at least three locations.

According to another aspect of the present invention, there is provided a system for warming cryopreserved liquid biological material disposed in a bag, during which the material changes its state from solid into liquid, the system comprising:

- a heat source;
- a warming device having a space for placing said bag therein, connected to the heat source and adapted to transfer heat from the heat source to the bag; and
- means for maintaining the heat source in heat transfer contact with a solid portion of said material to allow receiving said heat by said solid portion.

Disclosed below is an embodiment of such system also referred to as dry thawing device, a "DT device" or simply as "DTD". One of the benefits of using a dry device (in this and other aspects of the invention) is that there is no need to manage a heating fluid (e.g. water).

The system may comprise an empty space for accommodating any liquefied material apart from the cryopreserved material, which space may be a part of the bag spaced from the part where the solid portion of the cryopreserved material is located in heat transfer contact with the heat source. The liquefied portion of the biological material may be forced away from the area of said contact by suitable means, including for example a vacuum (applied within the bag), gravity, centrifugation, osmotic pressure, spring pressure and/or pneumatic or hydraulic pressure and any combination thereof.

Any one or more of the following features may be included in this aspect of the invention:

- The warming device may comprise a pair of plates with a suitable thermal mass, creating therebetween said space for the bag, at least one of said plates may be used as the heat source. The plates may be adapted for sandwiching an amount of cryopreserved (e.g. frozen) biological material therebetween to provide a predetermined desired temperature gradient to generate a high warming (e.g. thawing) rate.
- At least one of the plates may be further adapted to move with respect to the other plate in a direction perpendicular to a longitudinal axis of said bag, thereby applying pressure on the bag.
- The heat source may be adapted to continually supply heat to the warming device until all the cryopreserved material is liquefied and removed.
- The warming device may be adapted to store heat supplied by the heat source.
- The warming device may comprise heat storage means and the heat source is adapted to supply heat to said heat storage means of the warming device only prior to the warming process, during which the heat stored in the warming device is supplied to the bag.
- The bag may have a cryopreserved material section filled with the cryopreserved material before said warming and an empty section constituting said empty space, which is free of the cryopreserved material and is adapted to accommodate said liquefied material.
- The heat source may be an electrical heater.
- The device may be portable. It may be sufficiently light weight and be able to perform adequately using a portable energy source.

The system is adapted for applying a suitable mechanical pressure onto the bag, which can urge any liquefied material to a volume outside of or away from the frozen contents of the bag, to maintain substantially constant the thermal contact between the heated plates and the cryopreserved material via the bag walls only, without any substantial interference from liquefied thawed material and without overheating the biological material.

This aspect takes advantage, among other things, of the relatively better heat conductivity of solids, when compared with liquids.

According to another aspect of the present invention, there is provided a method for warming a cryopreserved liquid biological material disposed in a bag, the method comprising:

- providing a heat source;
- providing a warming device and placing said bag within a space therein;
- connecting the thawing device to the heat source adapted to transfer heat from the heat source to the bag; and
- removing from the bag a thawed material thereby preventing it from receiving said heat during warming of cryopreserved material left in the bag.

A system for performing the above method may also be referred to as dry thawing device, a "DT device" or simply as "DTD".

According to another aspect of the present invention, there is provided a system for warming a cryopreserved liquid biological material disposed in a bag having a longitudinal axis, the system comprising:

- a heat source;
- a warming device having a space for placing said bag therein and fluid accommodating means for accommodating a fluid therein, the device being adapted to transfer heat from the fluid to the bag; and
- means for maintaining a liquefied material within the bag evenly distributed along said longitudinal axis thereby improving heat transfer between said fluid and said cryopreserved material.

Such system may also referred to as a wet thawing device , "forced flow thawing device", "FT device", "Activator" "FFTD or "WTD".

Any one or more of the following features may be included in this aspect of the invention:

- The warming device may comprise a pair of plates creating therebetween said space for the bag.
- The fluid accommodating means may be channels comprised within at least one of said plates. The channels, such as heat transfer passages, may provide direct thermal contact between the fluid and the bag, and between the bag and those portions of the plates that are between the heat transfer passages.

- At least one of said plates may be adapted to move with respect to the other plate in a direction perpendicular to said axis, thereby applying pressure on the bag.
- The device may comprise an empty space for accommodating of any liquefied material apart from the cryopreserved material. Thus the device may prevent an increase of the surface-to-volume ratio during warming, by ensuring a relatively constant width of the bag, perpendicular to the longitudinal axis of the bag. Such increase of the surface-to-volume ratio during warming may occur when, instead of the warming device of the present invention, biological material in a bag is thawed directly in a tank filled with heating fluid, for example due to accumulation of the liquefied portion of the biological sample during warming (typically in the area of the bottom of the bag).
- The system may comprise, in addition to the warming device, an immersion tank filled with said fluid.
- The system may further comprise a pump for pushing said fluid from said tank to said fluid accommodating means.
- The fluid may be warm water.

The warming device may be adapted for applying a suitable mechanical pressure onto the bag, which at times removes liquified material to a volume outside of or away from the cryopreserved contents of the bag. For example, the plates may be mounted on springs which urge the plates towards the bag; as the material thaws, the bag presents less resistance to the plates, which effectively move closer the cryopreserved bulk within the bag, and a portion of the liquified contents thereof may be squeezed out to the upper extremity of the bag away from the cryopreserved core, and/or the bag walls may deform and bulge into the heat transfer passages, with liquefied material occupying these bulges rather than accumulating at the bottom of the bag and minimizing thermal transfer to any cryopreserved material thereat. This serves to maintain substantially constant the thermal contact between the heat transfer plates and the cryopreserved material still in the bag via the bag walls only, without any substantial interference from bulk liquified material.

According to another aspect of the present invention, there is provided a method for warming a cryopreserved liquid biological material disposed in a bag having a longitudinal axis, the method comprising:

- providing a heat source;
- providing a warming device having a space for placing said bag therein and fluid accommodating means for accommodating a fluid therein, for transferring heat from the fluid to the bag;
- providing means for maintaining a liquefied material within the bag evenly distributed along said longitudinal axis, thereby improving heat transfer between said fluid and said cryopreserved material; and
- removing said liquefied material from the heated portion of the bag (to other part of the bag or to a different bag) thereby preventing it from receiving said heat.

The method may be performed using a wet thawing device, "forced flow thawing device", "FT device", "Activator" "FFTD or "WTD" according to one or more embodiments of the invention.

In warming biological material of significant bulk, the liquefied portions of the material may insulate the portions of the material that are still cryopreserved (solid) and thus reduce the warming rate of the solid portion. Also, the thermal contact between the liquefied portion and the heat source may cause overheating of portions of the biological material. Thus, according to some aspects of the invention, thawing is carried out in a relatively rapid rate and in a manner to minimize recrystallization, which can otherwise occur due to the endothermic effect of thawing, whereby a thawing crystal may cause surrounding water to recrystallize. At the same time, thermal contact between the heat source used for warming and the cryopreserved part of the biological material is maximized during warming, thereby minimizing negative effects that may occur due to slow warming (or thawing). Additionally overheating of the biological material may be avoided or eliminated, for example, by removal of liquefied material from the thermal contact areas, and/or preventing accumulation of liquefied material at locations in the bag.

A system for cryopreserving and a system for warming biological materials, according to any of the embodiments of the present invention, may be provided as a kit.

It is to be noted that warming and/or cryopreserving according to aspects of the present invention of more than one sample of biological material may be performed in sequence or simultaneously. For cryopreserving for example, more than one bag may be placed in a cartridge (e.g. side by side) or multiple cartridges may be inserted simultaneously (e.g. one next to the other) or in sequence (e.g. one above the other) with respect to a single cold temperature source. For warming, more than one bag may be placed between the heat transfer plates (e.g. side by side), for example.

BRIEF DESCRIPTION OF THE DRAWINGS

In order to understand the invention and to see how it may be carried out in practice, embodiments will now be described, by way of non-limiting example only, with reference to the accompanying drawings, in which:

Figs. 1A to **1D** show the effect of cooling rate on homogeneity of freezing provided with a device according to one embodiment of the invention, as obtained in Experiment No. 4.

Fig. 2 schematically illustrates an embodiment of a freezing system according to one embodiment of the invention.

Fig. 3 schematically illustrates in isometric view an embodiment of a cartridge for use with the system of Fig. 2.

Figs. 4 and **4a** schematically illustrate, in isometric view and side view respectively, another embodiment of a cartridge for use with the system of Fig. 2.

Figs 5a and **5b** illustrate in plan view and side view, respectively, an embodiment of a freezing and thawing bag for use with the embodiments of Figs. 1 to 4, 7, 9a and 9b.

Figs. 6a and **6b** compare temperature gradient effects obtained with and without a temperature controlled zone above the cold temperature source.

Fig. 7 schematically illustrates an embodiment of a thawing system according to one aspect of the invention.

Fig. 8 illustrates in plan view another embodiment of a freezing and thawing bag for use with the embodiments of Figs. 1 to 4, 7, 9a and 9b.

Figs. 9a and **9b** schematically illustrate another embodiment of a thawing system according to one aspect of the invention.

Fig. 10 illustrates results obtained in Experiment 8.

Fig. 11 is a schematic representation of a desired cooling behavior according to some embodiments of the invention. The chart depicts the temperature (T) taken at different times (t) during immersion of the bag. The temperature is measured at three different locations (channels) along the longitudinal axis of a bag containing biological material.

Fig. 12 is a chart depicting survival of fresh donkey RBC (closed circles) and freeze-thawed donkey RBC (open circles) during 24 hours after transfusion of FITC labeled cells into donkeys.

DETAILED DESCRIPTION OF EMBODIMENTS

According to one aspect of the invention, a system and method for cryopreserving (freezing or vitrifying) biological materials, for example blood and blood components according to some embodiments is provided. According to one embodiment, the system, generally designated herein with the numeral **100** and illustrated in Fig. **2**, comprises an apparatus or device **20** for providing a cold temperature source, including a feed mechanism **50** for immersing and retracting into the device **20** a cassette or cartridge **60** comprising a bag **90**, having a longitudinal axis **90a** containing the biological materials to be frozen.

The device **20** is in the form of liquid nitrogen (LN) dewar (or any other suitable cold temperature source), and comprises a vessel or tank **21**, having side walls and a base and defining a volume V containing LN at a temperature T_2 of about **-196°C** (or at any other suitable temperature, preferably at or below about -80°C), and a cover **25** having an inner surface 25a which faces the interior of the tank 21. The cover comprises a slot-like access opening **28** having a width dimension W smaller than a length dimension. Downwardly depending into volume V from the periphery of the opening **28** is a plurality of tongues **29**. The tongues **29** may be arranged in two sets, one along each of the opposite long sides (i.e., along the length direction) of the opening **28**. The tongues **29** have a proximal distance D_1 and a distal distance D_2 therebetween, so that $D_2 \leq D_1$. In addition, the distal distance D_2 is equal or lesser than the width dimension W of the opening **28**, i.e. $D_2 \leq W$. One reason for the above relations is that the frozen bag should be removed; therefore it can not be wider than the opening width W. The lower ends of the tongues may be joined to a lower frame member **27** to prevent or minimize the ends separating when the system is in operation.

The tank 21 may further comprise suitable maximum and minimum level sensors, LS_{max} and LS_{min} for monitoring the level of LN in the tank **21** (although the device may be operated with only one sensor or with no sensors). When the level of LN drops below the minimum measured by LS_{min}, an alarm may be generated, advising the user to top up the tank **21** with more LN, and/or suitable means may be provided for automatically feeding the tank **21** from a suitable LN source. The other sensor LS_{max} advises when the maximum level has been reached, and to discontinue filing. Additionally or alternatively, filling may be discontinued at a predetermined time or after a predetermined volume has been added, without the use of an additional sensor. If the level LS_{max} is exceeded, it may be required to remove some LN, for example via natural evaporation (e.g. opening the cover **25**) and/or via a tank drain (not shown). Above the level of LN in the tank **21** a headspace may be provided. LN level may be measured using any one of known methods, for example including use of thermocouples, by weighing, and by measuring conductivity. One commercially available sensor that may be used in the present invention is LS2 Liquid Nitrogen Sensor (Teragon, USA).

Referring to Fig. **3,** in one embodiment the cartridge **60** comprises a pair of frame members **61, 62,** hinged together at a common pivot axis via hinge **63** at the lower ends thereof, enabling the frames to pivot with respect to one another from a closed position illustrated in Figs. **2** and **3,** to an open position. The frame members **61, 62** are similar in size and shape, which may be rectangular as illustrated, or any other suitable shape. Thus, frame member **61** comprises an opening A defined by side elements **61**b, **61**c, bottom element **61**a, and top element **61**d, and frame member **62** similarly has an another opening B (not seen in the figures) defined by side elements (only **62**c visible in Fig **3**), bottom element **62**a, and top element **62**d.

Each of the frame members **61, 62** comprises a wall **30** spanning the openings A, B, respectively. Preferably, these walls are formed of thermally conductive material, and are perforated, to increase heat transfer from the biological material to the cold temperature source. Suitable holes of other perforations (schematically shown at X) in the walls **30**, allow fluid communication between the outside of the cartridge, through the walls **30**, and into the space between the walls **30** of the two frame members **61, 62.** The perforated walls **30** are each designed to provide a structure of relatively high rigidity, and also to provide good fluid communication therethrough. The open area

provided by the perforations can be maximized while maintaining sufficient rigidity in the walls **30** to contain the shape of a bag **90** placed between the walls **30** when the bag is frozen. By way of non limiting example, the open area of the perforations may comprise between **60%** and about **70%**, say **63%**, of the plan area of the corresponding wall.

Frame **62** further optionally comprises a handle element **64** extending from top element **62**d in a direction away from the bottom element **62**a; alternatively or additionally, frame member **61** may comprise a similar handle. In the closed position, the frames are spaced from one another by a spacing, which is adapted for receiving and holding therein a bag **90**, and the frames can be locked in this position by means of one or more closure members **65**, **66**. A pair of closure members **65** may be provided, one each hinged at **69** on a different side of the cartridge **60** to one of side elements of the frame members **61** (or alternatively of frame member **62**), and each comprising a U-shaped cross-section and extended sides that retain frame members **61**, **62** along the length thereof in the closed position. When the closure members **65** are swung about their corresponding hinges **69**, the extended sides thereof come out of engagement with the frames **61**, **62**, which may then open to the open position. Closure member **66** is similar to closure members **65**, and is hingedly mounted to a bottom element of frame member **61** (or alternatively to a bottom element of frame member **62**). Alternatively, any other suitable closure members, including clamps or belts for example, or other arrangement may be used for enabling the frame members to reversibly close, to define the aforesaid spacing, and open to allow a bag to be inserted therebetween or removed therefrom.

The cartridge **60** optionally or alternatively further comprises a plurality of stiffening members **67**, joined at their ends to the top element **61d** and bottom element **61a** of frame member **61**. Each stiffening member **67** may comprise a U cross section, open at the longitudinal ends **38** thereof, and comprises a plurality of through holes **68**. The stiffening members are intended to keep a width of the bag uniform.

The cartridge **60** optionally or alternatively further comprises one or more separators, allowing an insertion of more than one bag, with each of the two outmost bags abutting one of the perforated walls. The separators may in a form of a flexible sheet, made of a having a little thermal mass, and optionally also being permeable to LN and/or having high heat transfer capability. Examples for such material inclde

polyurethane foam, thin aluminum sheets (perforated or not). The inventors found that use of such one or more separators according to an embodiment of the invention may reduce the post freezing thickness of the bag and improve cell post thaw viability. In addition, the post thaw viability may also be improved by decreasing the thickness of the cartridge and by inserting more than one bag thereinto, thereby decreasing the thickness of each bag.

Referring to Figs. 4 and 4a, another embodiment of the cartridge **60'** comprises all the elements and features of the embodiment of Fig. 3, *mutatis mutandis*, with some differences, as will be described herein. Thus, the cartridge **60'** comprises a pair of frame elements **61'**, **62'**, hinged together at **63'**, and each frame member **61'**, **62'** defining an opening, **A'** and **B'** respectively, comprising a perforated wall **30'**. Additionally, cartridge **60'** may each comprise one or more webs or rods **68'** on the top-inner sides of one or both walls **30**, optionally parallel to and below the corresponding top elements **61d'** and **62d'** of frame members **61'** and **62'**, respectively. These webs or rods **68'** may provide several functions. For example, when cartridge **60** is in the closed position, the rods may serve as spacer elements, to define spacing **S** between the walls **30** in which the bag **90** may be accommodated. In addition, the webs/rods **68'** may be configured for enabling the top portion of bag **90** to be tightly pressed between them when the cartridge is in the closed position, thus preventing crystal growth above the rods' level. Accordingly, when in use, the bag **90** may be placed in such manner that the portion of the bag immediately above the level of the biological material in the bag is tightly pressed between the rods **68'**. Furthermore, the frame members **61'**, **62'** may be held together in the closed position via latches **69'**, which may be provided on one of the handles **64a'** and designed to engage with respect to the other handle **64b'**.

Optionally, cartridge **60** may also comprise rods similar to rods **68'** described for cartridge **60'**, *mutatis mutandis*.

Referring to Figs. **5a** and **5b**, the bags **90** each comprise form or shape that is substantially complementary to that of the spacing between the frame members **61**, 62 of cartridge **60** (or frame members **61'**, **62'** of cartridge **60'**), and allows the bag to be accommodated therein when the cartridge is closed. Each bag may be made from a pair of durable flexible sheets **91**, **92**, having a rectangular or other plan form, and joined together at the peripheral edges **93**, to provide a containment volume Q between the sheets, which are spaced by a spacing S_0. One or more openings **95** may be provided,

for example formed as closable tubes between facing parts of said peripheral edges, to allow the bags to be filled or emptied. The bags **90** are also designed to provide a high surface area / volume ratio at a thin width or spacing S_0. By way of non limiting example, a bag **90** may have rectangular dimensions of about 265mm by about 360mm, and/or a holding volume of between about 300ml and about 500ml or 600ml, and/or a spacing S_0 of between about 4mm to about 12mm or to about 27mm or more (including for example between about 8mm to about 10mm).

The bags **90** may be made from any suitable biologically compatible material, and which preferably facilitates heat transfer between the inside and the outside of the bag **90**. To reduce or avoid damage to the bag during cryopreservation due to the expansion of the biological material, it is preferred that the material would have a glass transition temperature that is below that of the biological material. By way of non-limiting example, such bags **90** may be made from sheets of aluminium foil laminated with polyethylene, for example commercially available metalized polyethylene sheets (for example as purchased from Aran Packaging, Israel), or from sheets made from nylon and polyethylene, for example PerfecFlex 30652W, provided by PerfecSeal (UK).

Referring to Fig. **2**, the feed mechanism **50** is located above the tank **21**, and comprises a feeding passage **52** having an outlet **53** that is aligned with the slot-shaped opening **28**, and adapted for receiving therein a cartridge **60** via inlet **54**. Suitable drive means (not shown) are provided for translating the cartridge **60** along direction **P1** linearly along passage **52** and into the tank **21** via opening **58**, at a predetermined immersion velocity, **v** with respect to the tank **21**, and particularly the LN therein, as will be further described herein. The drive means also enable the cartridge with the bag to be removed from the tank **21** in direction **P2** after the freezing process is completed. Surrounding the passage **52** is a temperature control block or jacket **55**, which is adapted for setting the datum or start temperature of the bag generally independent of fluctuations in the external ambient temperature. The feed mechanism further comprises a heater **57**, for example an electrical heater, coupled to the jacket **55**, for establishing in the passage **52**, and particularly in a bag-containing cartridge **60** therein, a temperature T_1 greater than the freezing temperature of the biological material and optionally also above the ambient temperature T_0 arising above the cold temperature source. In order to set a desired temperature jacket **55** may have coupled to it also cooling elements and closed loop control using thermocouples, and other suitable features and components.

The system **100** may be operated as follows. A bag **90** is filled with a biological material, for example blood (optionally also comprising one or more added cryoprotectant agents), and closed in any suitable manner, for example vacuum sealed. The cartridge **60** is opened by releasing the closure members **65** and **66**, and pivoting frame members **61** and **62** away from one another about hinge **63**. The bag **90** is placed in the cartridge **60**, and the frame members pivoted back to the closed position, wherein the closure members are clamped to the closed position, trapping the bag **90** in the space between the frame members in a tight fitting manner and optionally having the top portion of the bag **90** tightly pressed between or under the frames' rods immediately above the level of the biological material when the cartridge **60** is thus configured. Most or a majority of the outer surface of each side **91**, **92** of the bag **90** are exposed via the perforations of the walls **30** covering openings A and B of the frames. The thus loaded cartridge **60** is then placed in the passage **52** via opening **54**, and driven towards the tank **21** along direction **P1**, the heater having heated at least the lower part of jacket **55** to a temperature T_1 above ambient T_0 and thus also the cartridge **60** (being in thermal contact with the jacket), the bag **90** with its contents. By way of non-limiting example, the bag is thus heated and maintained at a temperature of between **0°** and about **30°**, preferably between about room temperature (e.g. **20°**) to about **25°** prior to being immersed in the tank **21**.

The cartridge **60** is then lowered at a predetermined controlled velocity **v** into the volume **V** via the opening **28**. The drive means allow to select a predetermined value of the immersion velocity from plurality of values, and to maintain this value constant during the immersion of the cartridge **60**. The velocity is selected so as to ensure that cooling behavior of the biological material at least at three different locations along the direction P1 is as shown in Fig. 11 for three different channels C1, C2 and C3. Fig. 11 shows that in a range of temperatures T within time t the cooling behavior is defined by a T(t) curve having initial curved portion 11A, central essentially linear portion 11B and final curved portion 11C, wherein the central portions correspond to more than half of the range of temperatures, and at least the central portions for all the three channels are parallel.

The tongues **29** act as guides, for example rail guides, for the cartridge **60**, as this is being lowered into the LN-containing volume **V**. The tongues **29** engage the corresponding walls **30** of the cartridge and/or the stiffening members **67**, so as to abut

and press against them and prevent the bag from increasing in volume and/or bulging at any point.

This arrangement ensures that the bags have an essentially constant width S_0 throughout, minimizing the bulging that otherwise accompanies material frozen in bags.

Optionally, the tongues may be replaced with a cage or other arrangement that presses the bags to keep and maintain a substantially uniform thickness when held vertically, and while maximising the direct fluid communication between the LN and the bag. Thus, in other embodiments of the invention, the tongues may be replaced with perforated double wall structures, while the frame members are provided without walls **30**, so that the bag **90** in the cartridge has substantially fully exposed sides via opening A, B. When immersed in the tank **21**, the sides of the bag are in thermal contact with these perforated walls and thus the LN (in relative terms, in a similar manner to the embodiment described above having the perforated walls on the cartridge).

The construction of the cartridge **60** and the tongues **29** is such as to maximize direct thermal contact between the LN in volume V and the bag **90**. The tank **21** contains a sufficiently large volume of LN to enable it to absorb substantially enough the heat from the biological sample to enable it to freeze, without reducing the level of LN below a predetermined limit. Any LN that evaporates away is replaced, either manually or automatically, and the level is maintained almost constant by monitoring with level sensors LS_{max} and LS_{min}. Optionally, the minimal level of cooling fluid is the tank is no more than 2 cm (or 1 inch) below the maximal level of the cooling fluid.

The structure and operation of the system with respect to cartridge **60'** is similar to that described herein for cartridge **60**, *mutatis mutandis*.

Without being restricted to theory, inventors consider that as the cartridge is immersed into the LN, the sharp temperature gradient between the LN and the temperature controlled jacket **55**, which is kept in close proximity to the cover **25**, results in reproducible freezing conditions and together with the controlled velocity for immersion results in ice crystals growing quickly from the outer walls towards the inner part of the bag in a direction having significant lateral and upwards components (Fig. **6a**).

The immersion velocity **v** is such as to provide as uniform as possible a cooling rate for different parts of the bag **90**, while minimizing exothermic reaction effects. The faster the immersion velocity **v**, the faster LN is evaporated around the bag, which may

cause nitrogen gas to form around the bag in bubbles, temporarily reducing the heat transfer from the bag, and introducing chaotic cooling rates in the bag. On the other hand, slowing the rate R results in a longer the exothermic period (since the cooling rate is limited by the velocity of immersion); and the longer the exothermic period, the more damage than can occur to cells, blood cells for example, when these are comprised in the biological material. By way of non limiting example, an immersion velocity **v** of between about 1mm per second and about 3mm per second may be optimal. The immersion velocity **v** may vary with the thickness of the bag **90**, and thus also indirectly with respect to the bag surface and volume.

At times, when the preferred cooling rate is high (above 5°C) and the biological sample is bulky, it is desired to maintain a reduced width of the bag (perpendicular to the longitudinal axis) and to set the velocity of immersion such that it will be proportional to the rate of heat removal from the bag (e.g. less than 3mm/sec or even less than 1mm/sec). This may provide essentially identical cooling rates for different portions of the biological material. A faster immersion velocity would lead to higher boiling of LN. This would mean that the leading end of the bag would experience a higher temperature difference and hence have a higher cooling rate than the middle and tailing portions of the bag.

According to another aspect of the invention, a device and method for warming (e.g. thawing) biological materials, for example blood and blood components according to some embodiments, is provided.

According to one embodiment, the device, generally designated herein with the numeral **200** and illustrated in Fig. **7**, comprises a housing **210** having an upper part **214** and a lower part **212**, respectively accommodating an upper heating plate **234** and a lower heating plate **232**. The upper part **214** is displaceable with respect to the lower part **212**, for example by means of a hinge arrangement, between an open position, in which a frozen bag **90** may be placed over the lower heating plate **232**, and a closed position in which the plates **232, 234** are in generally overlying relationship sandwiching the bag **90** therebetween. Each plate **232, 234** comprises a pad **222, 224**, respectively having a high thermal mass, and a heating element, **252, 254**, respectively, for example electrical heaters, for heating the respective pads **222, 224**. Suitable springs **270** are provided between each plate **232, 234** and the respective casing part **212, 214**, that provide a mechanical pressure force onto the bags when this is in the device **200**

when in the closed position. Alternatively, a pneumatic, hydraulic or other arrangement may be provided to generate this pressure force. A ramp **260** (in this example, a downwardly sloping ramp) is provided on the bottom part **212** for supporting an empty (vacuumed) part **99** of the bag **90**, i.e. a part thereof in which no biological material was cryopreserved. Alternatively, a conduit leading from the bag interior may be supported by the ramp, the conduit being connected to a vessel or other bag to collect the liquefied contents directly.

In operation, the heaters **252, 254** heat the pads **222, 224,** to a suitable temperature T_w, which by way of non-limiting example may be between about 50°C and about 90°C, preferably about 70°C, and sufficient to thaw a particular volume (or width) in a desired time period. A bag **90** is sandwiched between the pads **222, 224,** with the empty (vacuumed) part projecting from the device **200** and resting on ramp **260.** As the pads **222, 224** heat the bag, the weight of the upper plate **234** and part **214,** together with the force exerted by springs **270** force the thawed material away from the space between the plates **232, 234,** and via the ramp towards the empty part **99** of the bag or to a waiting vessel, for example. By taking thawed material away from the bag volume continuously, thermal (heat transfer) contact between the frozen contents of the bag and the heated pads **222, 224** is maintained (or even maximized) and thermal contact between the thawed contents and the pads is reduced.

Optionally, the pads **222, 224** may be continually heated by the heaters **252, 254** during the thawing operation. Alternatively, the pads **222, 224** may have a higher thermal mass and are heated only prior to the thawing operation, and thawing progresses due to the stored heat in the pads **222, 224.**

By way of non-limiting example, a bag filled 500ml of cryopresereved biological material may be thawed in about 1 to about 3 minutes using a device **200** having preheated pads at a temperature of about 70°C. Typically, a sample of biological material having a width of 10-15 mm after it has been frozen, will be thawed in about 1 to 3 minutes at a temperature of about 70°C.

Referring to Fig 8, a variation of a freezing bag, designated **90'**, is illustrated, having a freezing compartment **192** and a collection compartment **194,** joined via a communicating passage **195,** but otherwise similar to bag **90** as described above, *mutatis mutandis*, having at least one inlet tube **197** to the freezing compartment. Other mechanisms for removal of the thawed portion of the biological material are mentioned

above. When freezing, the collection compartment is sealed off via a valve **196** or by clamping shut the passage **195,** for example using an external clamp or via the internal clamping action of rods such as rods **68'** when the cartridge is fitted with such rods, and the biological material is introduced into the freezing compartment **192** only. The bag **190** and contents are then frozen. When thawing, the whole bag, or just the freezing compartment **192** is placed between the plates **232, 234,** and the passage **195** opened, thereby enabling the thawed contents to be received in the collection compartment **194,** while enabling the thermal contact between the pads and the frozen contents, via the bag walls, to be maximized.

Optionally, a filter or mesh (not shown) may be provided in device **200** for preventing small frozen fragments from leaving the bag volume that is being heated by the pads.

Optionally, the device **200** may comprise one or more safety features, such as for example: a lock to prevent premature opening of the device, before operation (to assure a desired temperature before warming) and/or during operation (until a preset time sufficient to warm a bag); a detector for detecting that a bag is actually in place at the warming location between the pads; displays showing the temperature of the pads and/or of the bag; alarms for indicating that the thawing cycle has finished, and/or for alerting that the pad temperature has not reached, or is exceeding the desired temperature; and so on.

According to another embodiment, the warming device, generally designated herein with the numeral **300** and illustrated in Figs. 9a and 9b, comprises a housing **310** defining an immersion tank **312** and optionally a cover **314,** and an integrated unit **350** comprising a heat exchanger **320** in fluid communication with pump unit **340.** The heat exchanger **320** comprises opposed vertically arranged heat exchanger plates **322, 324,** defining a holding space M therebetween. The tank **312** comprises a duty fluid such as water, capable of heat transfer at desired rates.

The plates **322, 324** each have a plurality of facing open channels **332, 334,** respectively, having open inlet openings and outlet openings allowing free passage of a fluid medium therethrough. In the illustrated embodiment, the passages **332, 334** are shown as vertical, but in other embodiments the passages may have any suitable orientation. The plates **322, 324** are accommodated in a housing **330,** plate **322** statically, while plate **324** is horizontally movable towards and away from plate **322,** but

is urged towards the static plate **322** by means of springs **335** or any other suitable arrangement that can provide this effect. A suitable pump **360**, which may be located inside the housing or outside thereof, comprises an outlet **362** in fluid communication with an inlet end **332** of the housing **310**, aligned with inlet openings of the channels **332, 334,** via conduit **364.**

Holding space M is configured for vertically accommodating therein a bag **90** containing cryopreserved material, e.g. frozen biological material, such that the channels **332, 334** are facing the walls **91, 92,** respectively of the bag.

In operation, the water in tank **312** is suitably heated, for example by means of an electric heater (not shown) to attain a generally uniform temperature of, say between about **22**°C and about **60**°C, and preferably between about **37**°C and about **45**°C, by way of non-limiting example. A bag **90** with cryopreserved (e.g. frozen) contents is vertically inserted between the plates **322, 324,** the movable plate **324** having first been separated against the springs **335.** As water is pumped through the channels **332, 334** by means of pump **360,** which has a pump inlet (not shown) in fluid communication with the water in tank **312,** heat in the water is transferred directly to the bag and its contents, via the surface of the bag facing the channels, and indirectly, through the contact with the blocks between the channels, enabling the frozen contents to begin to thaw. As the material in the bag thaws, there is less resistance by the bag to the force provided by the springs **335,** which force the plate **324** towards the static plate **322.** Thawed material is pushed upwards to the empty space **99,** and the channel form of the plates cause a bulging of the side walls of the bag into the channel, the bulging portions of the bag filling with thawed material. By pressing the two sides of the bag closer together and preventing buildup of thawed material at the bottom of the bag by means of the pads **322, 324,** thermal contact between the frozen contents of the bag and the heat exchange channels **332, 334** is maximized.

Alternatively, the two chambered bag **90'** of Fig. 8 may be used with the embodiment of Figs 9a and 9b instead of bag **90**, in a manner similar to that described with respect to the embodiment of Fig 7, *mutatis mutandis*.

EXPERIMENTAL RESULTS

Terms and Abbreviations:

EGCG – Epigallocatechin gallate, a green tea catechin. Purchased from Zhejiang Yixin Pharmaceutical Co., Ltd.

LN – liquid nitrogen

IMT-1 – A freezing solution composed of 20% (w/v) Dextran 40 (Pharmacosmos, Denmark) and 0.945mg/ml EGCG dissolved in saline (0.9%(w/v) sodium chloride in double distilled water (DDW))

MCV – Mean corpuscular volume, this is a value measured by the automatic cell counter as part of the complete blood count (CBC) and gives an insight to the RBC volume.

Aluminum/PE bag – A bag made of aluminum foil (metallized polyethylene (PE). The aluminum/PE raw material is manufactured by Kolon Industries Inc., South Korea and laminated by Aran Packaging, Israel.

Cryoprotectant agent - denotes any agent that is added to a solution it improves the post cryopreservation viability (i.e. after thawing or liquefying) of a biological material cryopreserved in that solution. Intracellular CPAs are thought to replace water inside the cells, thus preventing crystallization therein, to enlarge the un-frozen fraction of the frozen solution, to buffer osmolarity and/or to stabilize the membrane and prevent mechanical damage caused by ice crystals. Examples of CPAs are DMSO, glycerol, ethylene glycol, poly ethylene glycol, propylene glycol, sugars, such as sucrose, dextrose, trehalose, and proteins, carbohydrates such as hydroxy ethyl starch (HES), dextran, etc.

Examples

Material and methods:

Except where indicated otherwise, in the following experiments the following materials and methods were used:

Packed RBCs from several donors (received from the Israeli Blood Bank, Magen David Adom) were pooled, and mixed with IMT-1 freezing solution in a 1:1 (v/v) ratio. It is noted that the freezing solution may be added to the RBC or the RBC added to the freezing solution, with comparable results (not shown). Before freezing, but after adding a freezing solution, a sample of blood was taken to be used as a control ("fresh").

For freezing, blood mixed with IMT-1 freezing solution was placed in freezing bags, and each bag was frozen in one of the following methods: (1) Submersion Freezing Device (SFD) – the bag was placed in a frame and the frame was lowered by the device into LN at a controlled velocity of 1mm/sec, (2) manual insertion into LN of a "naked bag" (i.e. without a freezing frame) and (c) slow freezing by placing the samples in a freezing frame, in a -80°C conventional freezer (Forma, USA) until thoroughly frozen. For LN freezing, the bag was held in LN for one minute after insertion, to ensure complete freezing. After freezing was complete, all samples were stored for 24 hours in the -80°C mechanical freezer (Forma, USA).

Thawing was done in as follows: The bags were removed from the freezing frame (if a frame was used) and their thickness was measured and then immediately thawed by one of the following: (1) placing the sample in an Activator (wet thawing device) or (2) by dipping samples in a still water bath, or (3) placing the sample in a DT device. Thawing was done, in each device, at a temperature of 22°, 37° or 45°C, as detailed in the experiment. The time needed for complete thawing was measured.

The blood was evaluated before freezing ("fresh") and after thawing for one or more of the following parameters:

- CBC – complete blood count using the Pentra 60 cell automatic cell counter (ABX, France). This indicates how many cells appear intact, regardless if they are also functionally intact. Unless otherwise specified the cell no. of thawed samples is presented as percent of the cell no. of the fresh (control) sample.

- Hematocrit (Hct) – was performed conventionally using microhematocrit capillaries and a microhematocrit centrifuge (sigma, USA). Calculation of Hct was done by dividing the packed RBC height in the total height received (packed RBCs and the supernatant) and multiplying by 100).

Supernatant free Hemoglobin (Hb) measurement were done by using the cyanmethemoglobin method using Drabkin's reagent (Sigma, USA) and reading absorbance at 540nm using an ELISA reader (EL-800, Bio-Tek Industries, USA).

Free Hb was calculated as follows:

$$\%\ Free\ Hb = 100X \frac{\text{(Absorbance of the supernatant)}}{\text{(Absorbance of supernatant + Absorbance of the pellet)}}$$

Experiment No. 1. **The effect of the method of freezing and the method of thawing on RBC recovery and the effect of the method of thawing on thawing time**

Specific details:

Samples were placed into 310X200mm aluminum/PE bags. Sample volume in each bag was 300ml. Each sample was a pool of blood from at least 3 different donors (all belong to the same blood group). Hb readings were performed in triplicates. Results are shown as the mean and standard deviation. The samples were frozen using the SFD or by manual insertion or by placing the sample in a mechanical -80°C freezer (Forma, USA).

In the following experiment frozen blood bags having a width of 6.5-9 mm were thawed either in the Activator or water bath, at 22°C, 37°C or 45°C.

Results:

A. Post freezing thickness -

Freezing in a Submersion freezing Device (6 bags), resulted in 8.38±0.38 mm thickness of the bag. Freezing by manual insertion to LN, without a freezing frame, resulted in 27±0 mm thickness (3 bags). Samples frozen in the mechanical -80°C freezer had a sample thickness of 7.66±1.1mm.

B. Thawing time

The thawing time results are depicted in Table 1. As seen, at any given temperature, the Activator thaws the sample much faster than the water bath. Additionally, for each thawing method the thawing time generally increases as the thawing temperature is lower.

Table 1. Thawing Time Using Activator or Water Bath at Different Temperatures of samples frozen using the SFD

Thawing temp (°C)	Thawing method	Sample thickness (mm)	Thawing time (sec)	Average Thawing Time
22	Activator	9	200	200
		9	200	
	water	8.3	420	510
		6.5	600	
37	Activator	8	140	107.5
		8.3	75	
	water	8.5	195	202.5
		6.5	210	
45	Activator	8	105	92.5
		8	80	
	water	8.5	160	160

C. RBC Recovery

Table 2 below depicts the MCV, cell number and hemoglobin assayed in thawed cells. As seen in Table 2, the MCV and cell number for cells frozen with SFD were about 100% (within the error margin of the Pentra device used to assay these parameters). Contrarily, when cells were frozen in a -80°C freezer, cells had 100% free hemoglobin regardless of the thawing conditions (water bath of Activator, at different temperatures) and appeared almost always as "all ghosts" (not shown) as can be seen in the samples that were manually frozen. These samples were inserted into LN gradually, similarly to the insertion by the SFD device (but without a metal frame, or controlled pre-freeze environment, or controlled velocity, etc.). As also seen in Table 2, sample thickness after freezing was highest in bags that were inserted manually..

Generally, as the thawing temperature increased the survival was higher, probably due to the fact that this allows faster thawing and as a consequence less damage from recrystallization.

Table 2. RBC Recovery After Freezing and Thawing at Different Conditions

Freezing method	Thawing method	Thawing temp (°C)	Thawing time (sec)	Free Hb (%) (Mean Value)	MCV (%)	Cell No (%)
SFD	Activator	45	105	3.98±1.16	99.22±1.78	98.66±1.67
SFD	Activator	37	140	4.21±1.57	98.06±0.67	99.52±0.24
SFD	water	45	160	4.58±0.3	97.65±0	97.54±0.4
SFD	water	37	195	5.35±0.4	98.04±0.7	99.29±1.23
SFD	Activator	22	200	12.49±0.9	100.40±0.70	98.41±0.77
SFD	water	22	420	15.30±0.73	102.41±2.09	101.92±1.3
Manual	water	45	410	10.93±1.2	103.92±0.7	97.91±0
Manual	water	37	540	17.98±0	108.24±0	99.08±0.6
Manual	water	22	900			All ghosts
-80°C freezer	Activator	22	200			All ghosts
-80°C freezer	Activator	37	75			All ghosts
-80°C freezer	Activator	45	80			All ghosts
-80°C freezer	water	22	600			All ghosts
-80°C freezer	water	37	210			All ghosts

Experiment No. 2. **The effect of the direction of immersion in LN on RBC Recovery after freezing in SFD**

Specific Details

Blood solution (packed RBC with IMT-1 freezing solution) was divided to four 300ml portions each placed in a 310mmX200mm aluminum/PE bag. The bags were frozen in SFD by submersion in LN at 1mm/sec in one of two directions: two bags were positioned vertically in respect to the LN (standing tall) and 2 were positioned horizontally (i.e. lying flat). Thawing was in the Activator device. Free Hb readings were performed, each repeated twice. Results are shown in Table 3 as the mean and standard deviation, compared to fresh blood.

Results:

Table 3. Summary of parameters and results

	Hb	Cell number (%)	MCV (%)
Horizontal	5.15±2.39	100.65±0.54	101.73±0.81
Vertical	2.82±0.75	100.78±0.36	99.43±0.81

Experiment No. 3. **RBC recovery after thawing in Activator or water bath**

Material and methods:

In this experiment RBC were frozen in 500ml volume of RBC/IMT-1 mixture in aluminum/PE bags (210x300mm) using SFD. Each bag contained RBC from a different donor. After freezing the bags (12) were stored for 24 hours in a -80°C freezer (Forma, USA). Each thawing condition was repeated in triplicates. Samples were thawed either in a regular water bath or in the activator at 37 and 45°C, each condition repeated thrice. The results are depicted in Table 4.

Table 4. Survival of frozen-thawed RBC

Temperature (°C)	Cell number (%)	MCV (%)	Free Hb (%)
Water bath 45°C	98.58±0.87	101.63±1.89	7.11±1.64
Water bath 37°C	99.0±1.7	102.68±0.6	10.49±3.2
Activator 45°C	100.2±0.7	103.45±5.0	6.27±0.8
Activator 37°C	98.8±0.9	99.22±1.8	7.27±2.6

As seen in Table 4, cell numbers and MCV were about the same for all samples, being within the margin of error for the assay. In terms of free Hb the survival of RBC after being thawed in the activator were slightly improved using the Activator. However, the standard deviation of the free Hb levels, when thawed in a regular water bath, was higher, indicating a higher variability between samples.

Additionally, when looking at the thawing time (Table 1 above), we can see it was much longer in a regular water bath compared to the activator at any given temperature. As expected, the decrease in temperature to 37°C from 45 °C increased thawing time in both cases. However, this increase in thawing time was significantly larger for the water bath than for the Activator. In the present experiment this difference was even higher, with the thawing time measuring about 2 minutes for the water bath, compared to only 30 seconds for the Activator (data not shown), although the samples thawed in the Activator were slightly thicker. It is therefore concluded that thawing in the Activator is less prone to be affected by slight experimental variation

(e.g. of the thawing temperature or other process steps, solution composition, etc.) than simple thawing in a water bath. This provides a thawing method with improved dependability.

Experiment No. 4. **The effect of the cooling rate on the homogeneity of SFD freezing**

Specific Details:

Aluminum/PE bags were filled with 300ml of IMT-1 solution (no RBC). 5 thermocouples (Pico, UK) (connected to a PC for data recording) were place along the longitudinal axis of the freezing bag, and the bags were frozen in SFD, each in one of the following velocities: 0.5, 1, 3 and 6mm/sec. Each freezing velocity was repeated 3 times, and the temperature was measured by the thermocouples continuously. All samples were frozen to a thickness of 9mm. The results are shown in **Figures 1A-1D** in the attached file.

Comparing **Figures 1C and 1D**, it is seen that at 3mm/sec and 6mm/sec (respectively) the measured cooling rates within the sample become less uniform. Due to the fact that the cryogenic fluid used was LN, the faster the velocity was the more LN boiling was caused. The boiling of LN is assumed to cause gas to temporarily form around the bag thus temporarily lowering the heat conductivity and the actual cooling rate, resulting ultimately in more chaotic cooling rates.

At a cooling rate of 0.5mm/sec (**Figure 1A**), the cooling rates along the sample were more uniform. However, quite a long exothermic reaction was observed when freezing is commenced. It is well established that the longer the exothermic period the more damage occurs to cells. At the velocity of 1mm/sec (**Figure 1B**) the cooling rates along the sample where most uniform and that the exothermic period was virtually nonexistent. In separate experiments (not shown) it was also observed that of these 4 velocities a cooling at 1mm/sec produces the highest RBC recovery rate.

Experiment No. 5. **Freezing survival of short dated (28 days) RBC**

Background

Short dated RBC (i.e. blood cells that were stored for some time; "sdRBC") are packed RBC units collected into CPDA-1 and stored for 28 days at 4°C. Sometimes there is need to preserve sdRBC (e.g. when rare blood type RBC is near expiry).

After such storage the sdRBC were treated as described above for fresh "new" packed RBC. 3 units, with a final volume of 300ml each, were prepared. One unit was pooled from two donors and two others were from a single donor each. Freezing was done using the SFD at 1 mm/sec. All bags were stored in -80°C overnight before thawing in the Activator at 45°C. Thawing time measured 75 seconds. Hct, cell count, MCV and free hemoglobin were measured and the results are shown in Table 5.

Table 5. Survival of sdRBC (28 days) after freezing in SFD and storage at -80°C

Donor #	Free Hb (%)	Hct		Cell no. (%)	MCV (%)
	Thaw	Fresh	Thaw		
1	2.98 ± 0.06	44.7	45	98.08	95.74
2	2.15 ± 0.23	51	54	97.72	96.43
Pool (1+2)	2.17 ± 0.12	47.1	49.5	98.21	97.73

It is known in the art that sdRBC have approximately the same amount of free Hb as freshly harvested RBC (with a slight immeasurable increase). As seen in Table 5, sdRBC survived freezing and thawing, resulting with only about 2-3% hemolysis (see free Hb values).

Experiment No. 6. **Freezing survival of short dated (34 days) RBC**

Specific Details:

The present experiment was conducted similarly to Experiment No. 5, with the following differences: Packed RBC (300ml) were provided by the Red Cross and stored in 4°C, for a total time of 34 days from the donation. The Red Cross in the USA usually collects blood into CPD containing bags. Later, the blood is centrifuged and ADSOL

(aka AS-1 and SAG-M) is added to the packed cells (pellet). These units of packed RBC (with ADSOL) can be stored in refrigeration (2-8°C) for up to 42 days. Each blood unit was mixed with 250ml IMT-1 freezing solution and transferred into a nylon/PE bag (total volume 550ml; produced from PerfecFlex 30652W, from PerfecSeal). The results are depicted in Table 6. Hct and MCV are presented as compared to the fresh control.

Table 6. Survival of sdRBC(34 days) after freezing in SFD and storage at -80°C

Bag No.	Freezing volume (ml)	fresh Cell concentration (· 10^6 cells/ ml)	Thawed Cell concentration (· 10^6 cells/ ml)	% Hct	% MCV	% free Hb in thawed units
990	550	3.29	3.33	109.68	101.28	3.3
992	580	3.28	3.33	101.76	103.57	3.7
204	550	3.46	3.49	111.52	102.38	3.2

As seen above, 34 days old sdRBC survived the prolonged storage followed by freezing (SFD, 1mm/sec), storage at 80°C and thawing (Activator 45°C). The cell count, MCV and Hct were normal. In addition, free Hb levels of thawed units were very low, indicating about 97% survival of RBC.

Experiment No. 7. **Effect of packed RBC storage solution and collection procedure**

Background:

The Red Cross normally collects blood into bags containing CPD (Anticoagulant Citrate Phosphate Dextrose USP containing Sodium Citrate (dihydrate) 26.3 g/L Dextrose (monohydrate) 25.5 g/L Citric Acid (anhydrous) 3.27 g/L and Monobasic Sodium Phosphate (monohydrate) 2.22 g/L). The blood is centrifuged and to the packed RBC AS-1 (ADSOL, also termed SAGM) is added (containing Sodium Citrate (dihydrate) 26.3 g/L, Dextrose (monohydrate) 25.5 g/L, Citric Acid (anhydrous) 3.27 g/L, Monobasic Sodium Phosphate (monohydrate) 2.22 g/L, Sodium Chloride, Mannitol and Adenine). Packed units that are with ADSOL can be stored up to 42 days in refrigeration. In Israel most of the donated blood is collected into bags containing CPDA-1 (Anticoagulant Citrate Phosphate Dextrose Adenine Solution USP Dextrose

(monohydrate) 31.9 g/L Sodium Citrate (dihydrate) 26.3 g/L Citric Acid (anhydrous) 3.27 g/L Monobasic Sodium Phosphate (monohydrate) 2.22 g/L and Adenine 0.275 g/L). packed RBC units in CPDA-1 can be stored up to 35 days in refrigeration.

Packed RBCs, received from the Red Cross (RC) and Magen David Adom (MDA) were each mixed in a ratio of 1:1 (v/v) with IMT-1 freezing solution to a final volume of 440ml. The mixture was transferred to a nylon/PE bag (produced from PerfecFlex 30652W, from PerfecSeal) and then frozen by the SFD at 1mm/sec. The bags were stored for 3 hours in a -80°C freezer before thawing. Thawing lasted about 120sec in an Activator. The results are depicted in Table 7, where Hct is depicted as percentage from fresh sample.

Table 7. Comparison of different storage solutions and collection procedures

Sample Source	Storage solution	Free Hb (%)	Cell number (%)	MCV (%)
RC880 (USA)	ADSOL	3.83±0.78	97.65	101.27
RC 529 (USA)	ADSOL	2.80±0.42	99.36	100.00
MDA 586 (Israel)	CPDA	2.28±0.75	100.24	97.92
MDA 587 (Israel)	ADSOL	3.18±0.36	99.68	100.00

The results in Table 7 show that there was no significant difference between the fresh and the post thaw cell count and MCV. In addition, when comparing the free Hb levels all samples showed very high survival rates (about 97-98% survival) with apparently no significant difference between the different storage conditions/solutions.

Experiment No. 8. Evaluation of post freezing storage on dry ice

Specific conditions:

A pool of 4 Packed RBCs units was used to produce 500ml units in four nylon/PE bags (produced from PerfecFlex 30652W, from PerfecSeal). Freezing was in SFD (1mm/sec). After freezing, the bag was immediately transferred to storage in dry ice for two hours and was then thawed. The three other bags were stored first in LN for two hours and then were transferred into dry ice for 19, 25 and 48 hr. Storage in dry ice was done in an expanded polystyrene box. The box contained 8 Kg dry ice placed

below the blood bags and 5Kg dry ice was placed on top of the frozen RBC bags. No ice was added during storage. Thawing of all the bags was done in the water bath activator (45°C).

Since during freezing ice was not added to the ice box, the temperature in the ice box was followed during storage by measuring the temperature of frozen dextran solution kept in dry ice in five different locations in the box. The results are shown in Figure 10. Measurement began when the group of three bags was inserted into the box.

As seen in Figure 10, during the first 20 hours, the temperature was almost without change, at about -77°C (with a slight increase of about 2°C during the last two hours). The first two bags (2 hours and 19 hours) were removed from the ice box during this period. At hour 25, when the third bag was removed the temperature increased to about -72°C. However, it is noted that by the this time a quick rate of warming was detected and by the time that the last bag was removed (48 hours) the temperature in the box was between -50°C and -55°C.

Upon removal from the ice box, the bags were thawed, and then assayed for MCV, Hct and free Hb. The results are shown in Table 8.

Table 8. RBC stored on dry ice after freezing in SFD

Sample	Cells concentration ($x10^9$/ml)	MCV	%Hct	% Free Hb
Fresh	2.89	89	25.7	0
Stored for 2hrs	2.88	88	25.3	1.84±0.64
Stored for 19hrs	2.87	88	25.4	1.35±0.92
Stored for 25hrs	2.81	88	24.8	1.85±0.07
Stored for 48hrs	2.86	88	25.2	6.03±0.04

As seen in Table 8, storage in dry ice has resulted in very high survival rates up to 25 hours with more then 98% RBCs survival (manifested by free hemoglobin values). During this time, the box temperature was between -72 and -77°C (below -70°C).An increase in hemolysis was detected at storage for 48 hours which resulted in about 94% RBC survival. This may be explained by the rise in temperature during the period between 25 and 47 hours of storage. The final temperature, in the vicinity of -50

- -55°C may be higher then the glass transition temperature of the sample causing recrystallization due to which there was increase in cells lysis.

Experiment No. 9. Effect of the composition of the freezing bag on RBC recovery

To date blood bags are normally manufactured of PVC. Such bags are not chilled below -80°C, and would not endure exposure to LN. Stem cells are frozen in EVA bags. These bags remain intact after freezing of up to 100ml preparations in LN.

The present inventors found that EVA bags holding more than 100 ml blood preparation tended to crack during freezing, thawing and/or storage. Therefore it was desirable to find a new material to prepare freezing bags. Three types of bags were used in various experiments. The bags were made to order from the materials as detailed in Table 9. 300 ml blood preparation (RBC/IMT-1 mixtures) were frozen in each bag.

It was found that 35785 bags tended to crack significantly more often thank the other two. Therefore Nylon/PE and Aluminum/PE bags are preferred. Aluminum/PE is also preferred for better conductivity and nylon/PE also preferred for transparence. Both aluminum/PE and nylon/PE bags provided acceptable RBC recovery (data not shown).

Table 9. Bag characterization:

Bag name	Bag description	Material
35785	Aluminum + EVA / PE	PerfecFlex 35785G, from Perfecseal
Nylon/PE	Nylon + PE	PerfecFlex 30652W, from Perfecseal
Aluminum/PE	Aluminum + PE	Metallized polyethylene, from AranPackaging

Experiment No 10. In vitro Evaluation of RBC thawed using Dry Thawing Device (DTD)

Samples comprising RBC and IMT-1 were prepared essentially as described above in Experiment 1 and placed into 310X200mm aluminum/PE bags. The average blood unit age before freezing was 5.67 days (SD=1.32). Sample volume in each bag was 300ml. A sample from each bag was taken before freezing (but after addition of

IMT-1 freezing solution) to serve as a "fresh" control. The samples were frozen using the SFD essentially as described above. After freezing was completed, the bags were transferred to a mechanical freezer for storage at -80°C (Forma, USA) for overnight storage.

Thawing was performed in a DTD, wherein the plates were heated to a temperature of 70°C before thawing, and heating was stopped as soon as the frozen bag was inserted to the device.

Table 10 compares RBCs parameters (Mean ± SD) of 9 units of RBCs before and after freeze-thawing. The control value for each measurement was obtained from samples taken from the same blood unit prior to freezing after the addition of IMT-1 solution (RBC+IMT-1 freezing solution).

Table 10. RBC properties before and after freeze-thawing

Parameter	treatment	Average± SD (n=9)
RBC (10^6/mm^3)	fresh	4.03±0.37
	Freeze-Thaw	4.02±0.37
Hgb (g/dL)	fresh	12.22±1.02
	Freeze-Thaw	12.28±1.02
HCT (%)	fresh	40.11±3.18
	Freeze-Thaw	39.11±2.07
MCV (μm^3)	fresh	85.33±2.60
	Freeze-Thaw	84.33±2.69
pH	fresh	7.08±0.02
	Freeze-Thaw	7.04±0.09
Plasma Hgb (%)	fresh	0.02±0.04
	Freeze-Thaw	0.52±0.10
Recovery (%)	fresh	99.86±0.24
	Freeze-Thaw	97.46±0.33

Experiment No 11. Freeze thawing RBC using DTD

Packed RBCs from 2 donors were mixed in a 1:1 ratio (v/v) with IMT-1 freezing solution. A nylon bag with 500ml was frozen using SFD. The post freeze thickness of the bags was 9mm. After freezing the bags were stored in a -80°C mechanical freezer (Forma, USA) for two hours. Thawing was done in the DTD set to 70°C before thawing, and thawing lasted 135 sec.

Cell count, mean corpuscular volume (MCV) and hematocrit were measured as described above. The level of free hemoglobin (Hb) was determined as well before and after freeze thawing using HemoCue plasma/low Hb photometer device (HemoCue AB, USA). The results are shown in Table11.

As seen in Table 11, in both donors cell count, hematocrit and MCV did not change significantly before and after thawing. In addition, free hemoglobin levels were very similar for the same freezing and thawing conditions resulting with about 3.5% free hemoglobin after freeze thawing.

Table 11. RBC properties before and after freeze-thawing

Donor	Before Freezing				After Thawing			
	Cell count ($X10^6$/ml)	MCV (μm^3)	HCT (%)	% hemolysis	Cell count ($x10^6$/ml)	MCV (μm^3)	HCT (%)	% hemolysis
1	3.05	85	31.3	0.07	3.04	87	31	3.45 ± 0.2
2	3.15	81	31.3	0.22	3.19	82	31	3.48±0.12

Experiment No. 12. Freezing and thawing a RBC sample at 3 mm thickness with low hematocrit

Packed RBC were mixed in a 1:2 ratio (v/v) with IMT-1 freezing solution. 250ml samples were placed in each nylon bag, and were frozen using the SFD, with the following modification.

Two bags were placed side by side in a single cartridge, each abutting an opposing perforated wall of the cartridge. The bags were separated by a 3 mm flexible

foam sheet of polyurethane. The post freeze thickness of each bag was 3mm. After freezing the bags were stored in a -80°C mechanical freezer (Forma, USA) for two hours. Thawing was done in the DTD set to 70°C before thawing.

Cell count, mean corpuscular volume (MCV), hematocrit and free hemoglobin (Hb) were determined as described in Experiment No. 11. The results are shown in Table 12. As seen in Table 12, the low hematocrit (20% or less or even 15% or less, or about 12-13%) improved post thaw viability. Similarly it is concluded that reducing the width of the bag to below 9mm (or even below 5mm, or to about 3mm) improved post thaw viability.

Table 12. RBC properties before and after freeze-thawing

Prior Freezing				After Thawing			
Cell con. ($\cdot 10^6$/ml)	MCV (μm^3)	HCT (%)	% hemolysis	Cell con. ($x10^6$/ml)	MCV (μm^3)	HCT (%)	% hemolysis
1.11	74	13	0	1.12	72	12	0.8

***Experiment No 13.* Survival of transfused freeze-thawed donkey RBC**

Donkey blood was obtained from "Segera Farm", Ilania, Israel. The blood was collected in a regular collection bag, containing CPDA-1 (MacoPharma, France). All donkeys transfusions were autologous.

A day before the transfusion, approximately 500mL of whole donkey blood was drawn out into CPDA-1 bags and sent to CD laboratories. The blood was centrifuged (4000g, 10min) and the supernatant plasma removed. The packed RBC were washed three times with saline-glucose buffer (1000g, 10min), and then mixed in a 1:1 ratio with the same buffer.

Donkey blood was labeled with Fluorescein isothiocyanate (FITC) (Sigma, USA), a common fluorescent dye, according to the protocol of Horie [Horie Y, Kato S, Ohki E, Hamamatsu H, Fukumura D, Kurose I, Suzuki H, Suematsu M, Miura S, Ishii H.: *Effect of lipopolysaccharides on erythrocyte flow velocity in rat liver,* J Gastroenterol., 783-90,;32(6): Dec, 1997]. After adding the FITC solution; the cells were gently shaken for 1 hour at room temperature and then washed four times with the

saline- glucose buffer. The saline-glucose buffer is comprised of 118.5 mM glucose, 3.68 mM Na barbiturate, 1.25 mM barbituratic acid, 0.55 mM Mg sulafate·7H2O, 0.15 mM CaCl·2H2O, and 58.2 mM NaCl.

When fresh (unfrozen) RBCs were to be transfused, the FITC stained RBCs were transferred to a sterile bag containing CPDA1, and kept at 4°C overnight. The next day the unit was transported on ice (approx. 5°C) to the farm and then transfused to the donkey over the course of an hour.

When frozen-thawed cells were to be transfused, the donkey's RBC were stained and then mixed in a 1:1 ratio with IMT-1 freezing solution, frozen essentially as described above, and stored overnight at -80°C. The following day the frozen sample was thawed using DTD as described in experiment 12, and its contents were transferred into a transfusion bag for administration to the donkey.

After completion of the transfusion, blood samples were drawn out from each donkey at the following time points: ten minutes and 2, 4 and 24 hours. The time point of 10 minutes after transfusion was used as the base line measurement (i.e. the concentration of labeled cells at that time was defined 100%).

RBC recovery and survival was determined by measuring the percent of stained cells out of the total RBC population. To that end, blood samples were taken from the animals in Vacutainer- K2EDTA tubes (BD, Franklin Lakes, NJ, USA). 20 µl of this blood were then suspended in 980 µl of Dulbecco's Modified Eagle's Medium (DMEM, 4500mg/l D-Glucose) (Biological Industries Inc., Beit-Haemek, Israel). Stained RBC were counted using a Fluorescence-Activated Cell Sorter (FACS), the number of stained and un-stained cells counted, and the ratio between them determined.

The results are depicted in **Fig. 12**, wherein it is seen that fresh stained blood and frozen thawed blood had similar survival patterns in the donor donkeys. Monitoring of the staining of donkey blood every several days for about 13 weeks after transfusion revealed that the staining pattern remained similar for both the fresh and frozen thawed transfusions (not shown).

CLAIMS:

1. A system for cryopreserving a liquid biological material disposed in a bag having a longitudinal axis, the system comprising:

- a bag holder for holding said bag, so that the biological material therein has a surface area S, and a volume V;
- a tank containing a cryogenic fluid;
- a mechanism for the immersion of said bag holder into said tank along said longitudinal axis;
- an opening in said tank for insertion therethrough of the bag holder; and
- a guide member extending from said opening into said tank and adapted for engaging walls of said bag holder, when the bag holder is being immersed into the tank via said opening, to reduce widening of the bag during its being frozen by the cryogenic fluid.

2. A system according to claim 1, comprising means to prevent ratio between that of the surface area S and that of the volume V of said biological material from increasing during the immersion to more than 4:3.

3. A system according to claims 1 or 2, wherein the guide member comprises a plurality of guides in the form of tongues depending into said tank from said opening.

4. A system according to any one of claims 1 to 3, wherein the guides are spaced by a proximal distance at a proximal portion of the guide member and a distal distance at a distal portion of the guide member, said distal distance being equal to or lesser than said proximal distance.

5. A system according to any one of claims 1 to 4, wherein said tank comprises a cover, wherein the proximal portion of the guide member is attached to said cover at the opening's periphery.

6. A system according to claim 5, wherein the cover has an inner surface facing the interior of the tank, and the proximal portion is attached to the inner surface.

7. A system according to any one of claims 1 to 6, comprising a means for keeping level of said cryogenic fluid in said tank within no less than 3 cm below the maximal fluid level during the immersion of the bag with the biological material.

8. A system according to any one of claims 1 to 7, wherein said tank comprises level sensors for monitoring a level of the cryogenic fluid within the tank.

9. A system according to any one of claims 1 to 8, wherein the bag holder is in the form of a cartridge comprising a pair of frame members.

10. A system according to claim 9, wherein said frame members constitute side walls of the cartridge, for placing the bag with the biological material in a space created therebetween.

11. A system according to any one of claims 1 to 10, wherein the bag holder comprises stiffening members for keeping a width of the bag uniform.

12. A system according to any one of claims 1 to 11, comprising a temperature control block for setting an immersion temperature of said bag, prior to said immersion, to a pre-determined value higher than a temperature of said cryogenic fluid.

13. A system according to claim 12, wherein said temperature control block is adapted for setting said immersion temperature to between about 0°C and about 30°C.

14. A system according to any one of claims 1 to 13, comprising a velocity control means allowing to select a pre-determined value of said immersion velocity and to maintain said pre-determined value constant during said immersion.

15. A system for cryopreserving a liquid biological material disposed in a bag having a longitudinal axis, the system comprising:

- a bag holder for holding said bag, so that the biological material therein has a surface area S, and a volume V;
- a tank containing a cryogenic fluid;
- a mechanism for the immersion of said bag holder into said tank along said longitudinal axis at an immersion velocity, wherein said immersion velocity may be of a plurality of values; and
- a velocity control means allowing to select a pre-determined value of said immersion velocity from said plurality of values, and to maintain said pre-determined value constant during said immersion.

16. A system according to claim 15, wherein said tank comprises an opening in said tank for insertion therethrough of the bag holder, at said immersion velocity, said opening having an opening width.

17. A system according to any one of claims 15 or 16, comprising means to prevent ratio between that of the surface area S and that of the volume V of said biological material from increasing during the immersion to more than 4:3.

18. A system according to any one of claims 16 or 17, comprising a guide member extending from said opening into said tank and adapted for engaging walls of said bag holder, when the bag holder is being immersed into the tank via said opening, to reduce widening of the bag during its being frozen by the cryogenic fluid.

19. A system according to Claim 18, wherein the guide member comprises a plurality of guides in the form of tongues depending into said tank from said opening.

20. A system according to claim 19, wherein the guides are spaced by a proximal distance at a proximal portion of the guide member and a distal distance at a distal portion of the guide member, said distal distance being equal to or lesser than said proximal distance.

21. A system according to any one of claims 18 to 20, wherein said tank comprises a cover, wherein the proximal portion of the guide member is attached to said cover at the opening's periphery.

22. A system according to claim 21, wherein the cover has an inner surface facing the interior of the tank, and the proximal portion is attached to the inner surface.

23. A system according to any one of claims 15 to 22, comprising a means for keeping level of said cryogenic fluid in said tank within no less than 3 cm below the maximal fluid level during the immersion of the bag with the biological material.

24. A system according any one of claims 15 to 23, wherein said tank comprises level sensors for monitoring a level of the cryogenic fluid within the tank.

25. A system according to any one of claims 15 to 24, wherein the bag holder is in the form of a cartridge comprising a pair of frame members.

26. A system according to claim 25, wherein said frame members constitute side walls of the cartridge, for placing the bag with the biological material in a space created therebetween.

27. A system according to claim 26, wherein the bag is of a shape substantially complementary to a shape of said space.

28. A system according to any one of claims 25 to 27, wherein the frame members are perforated to reduce the thermal mass of the frame.

29. A system according to any one of claims 15 to 28, wherein the bag holder comprises stiffening members for keeping a width of the bag uniform.

30. A system according to any one of claims 15 to 29, comprising a temperature control block for setting an immersion temperature of said bag, prior to said immersion, to a pre-determined value higher than a temperature of said cryogenic fluid.

31. A system according to claim 30, wherein said temperature control block is adapted for setting said immersion temperature to between about 0°C and about 30°C.

32. A system for cryopreserving a liquid biological material disposed in a bag having a longitudinal axis, the system comprising:

- a bag holder for holding said bag, so that the biological material therein has a surface area S, and a volume V;
- a tank containing a cryogenic fluid;
- a mechanism for the immersion of said bag holder into said tank along said longitudinal axis; and
- a temperature control setting an immersion temperature of said bag, prior to said immersion, to a pre-determined value higher than a temperature of said cryogenic fluid.

33. A system according to claim 32, temperature control block is adapted for setting said immersion temperature to between about 0°C and about 30°C.

34. A system according to any one of claims 32 or 33, wherein said tank comprises an opening in said tank for insertion therethrough of the bag holder, at said immersion velocity, said opening having an opening width.

35. A system according to any one of claims 32 to 34, comprising means to prevent ratio between that of the surface area S and that of the volume V of said biological material from increasing during the immersion to more than 4:3.

36. A system according to any one of claims 34 or 35, comprising a guide member extending from said opening into said tank and adapted for engaging walls of said bag holder, when the bag holder is being immersed into the tank via said opening, to reduce widening of the bag during its being frozen by the cryogenic fluid.

37. A system according to claim 36, wherein the guide member comprises a plurality of guides in the form of tongues depending into said tank from said opening.

38. A system according to claim 37, wherein the guides wherein the guides are spaced by a proximal distance at a proximal portion of the guide member and a distal distance at a distal portion of the guide member, said distal distance being equal to or lesser than said proximal distance.

39. A system according to any one of claims 36 to 38, wherein said tank comprises a cover, wherein the proximal portion of the guide member is attached to said cover at the opening's periphery.

40. A system according to claim 39, wherein the cover has an inner surface facing the interior of the tank, and the proximal portion is attached to the inner surface.

41. A system according to any one of claims 32 to 40, comprising means for keeping level of said cryogenic fluid in said tank within no less than 3 cm below the maximal fluid level during the immersion of the bag with the biological material.

42. A system according to any one of claims 32 to 41, wherein said tank comprises level sensors for monitoring a level of the cryogenic fluid within the tank.

43. A system according to any one of claims 32 to 42, wherein the bag holder is in the form of a cartridge comprising a pair of frame members.

44. A system according to claim 43, wherein said frame members constitute side walls of the cartridge, for placing the bag with the biological material in a space created therebetween.

45. A system according to claim 44, wherein the bag is of a shape substantially complementary to a shape of said space.

46. A system according to any one of claims 43 to 45, wherein the frame members are perforated to reduce the thermal mass of the frame.

47. A system according to any one of claims 43 to 46, wherein the frame members comprise a common pivot axis.

48. A system according to claim 47, wherein the frame members are adapted to move with respect to said pivot axis and with respect to each other, thereby changing a position of the bag holder between an open and a closed positions.

49. A system according to claim 48, wherein the bag holder, while in said closed position, has a shape that corresponds to a shape of the opening.

50. A system according to any one of claims 32 to 49, wherein the bag holder comprises stiffening members for keeping a width of the bag uniform.

51. A system according to any one of claims 32 to 50, wherein the cryogenic fluid is a liquid.

52. A system according to any one of claims 32 to 51, comprising a velocity control means allowing to select a pre-determined value of said immersion velocity, and to maintain said pre-determined value constant during said immersion.

53. A system for cryopreserving a liquid biological material disposed in a bag having a longitudinal axis, the system comprising:

- a bag holder for holding said bag, so that the biological material therein has a surface area S, and a volume V;
- a tank containing a cryogenic fluid;
- a mechanism for the immersion of said bag holder into said tank along said longitudinal axis; and
- means to prevent ratio between that of the surface area S and that of the volume V of said biological material from increasing during the immersion to more than 4:3.

54. A system according to claim 53, wherein said tank comprises an opening in said tank for insertion therethrough of the bag holder, at said immersion velocity, said opening having an opening width.

55. A system according to claim 54, comprising a guide member extending from said opening into said tank and adapted for engaging walls of said bag holder, when the bag holder is being immersed into the tank via said opening, to reduce widening of the bag during its being frozen by the cryogenic fluid.

56. A system according to claim 55, wherein the guide member comprises a plurality of guides in the form of tongues depending into said tank from said opening.

57. A system according to claim 56, wherein the guides wherein the guides are spaced by a proximal distance at a proximal portion of the guide member and a distal distance at a distal portion of the guide member, said distal distance being equal to or lesser than said proximal distance.

58. A system according to any one of claims 55 to 57, wherein said tank comprises a cover, wherein the proximal portion of the guide member is attached to said cover at the opening's periphery.

59. A system according to claim 58, wherein the cover has an inner surface facing the interior of the tank, and the proximal portion is attached to the inner surface.

60. A system according to any one of claims 53 to 59, a means for keeping level of said cryogenic fluid in said tank within no less than 3 cm below the maximal fluid level during the immersion of the bag with the biological material.

61. A system according to any one of claims 53 to 60, wherein said tank comprises level sensors for monitoring a level of the cryogenic fluid within the tank.

62. A system according to any one of claims 53 to 61, wherein the bag holder is in the form of a cartridge comprising a pair of frame members.

63. A system according to claim 62, wherein said frame members constitute side walls of the cartridge, for placing the bag with the biological material in a space created therebetween.

64. A system according to claim 63 wherein the bag is of a shape substantially complementary to a shape of said space.

65. A system according to any one of claims 62 to 64, wherein the frame members are perforated to reduce the thermal mass of the frame.

66. A system according to any one of claims 62 to 65, wherein the frame members comprise a common pivot axis.

67. A system according to claim 66, wherein the frame members are adapted to move with respect to said pivot axis and with respect to each other, thereby changing a position of the bag holder between an open and a closed positions.

68. A system according to claim 67, wherein the bag holder, while in said closed position, has a shape that corresponds to a shape of the opening.

69. A system according to any one of claims 53 to 68, wherein the bag holder comprises stiffening members for keeping a width of the bag uniform.

70. A system according to any one of claims 54 to 69, comprising a temperature control block for setting an immersion temperature of said bag, prior to said immersion, to a pre-determined value higher than a temperature of said cryogenic fluid.

71. A system according to claim 70, wherein said temperature control block is adapted for setting said immersion temperature to between about 0°C and about 30°C.

72. A system according to any one of claims 54 to 71, comprising a velocity control means allowing to select a pre-determined value of said immersion velocity, and to maintain said pre-determined value constant during said immersion.

73. A method for cryopreserving a liquid biological material disposed in a bag having a longitudinal axis, the method comprising:

- placing the bag in a bag holder; and
- immersing said bag holder into a cryogenic fluid in a direction parallel to said longitudinal axis at an invariant immersion velocity selected so as to ensure that the thermal curve of the biological material at least at three different

locations along the longitudinal axis of the bag is essentially as shown in Fig. 11.

74. A method according to claim 73, wherein said bag holder is immersed into a tank containing said cryogenic fluid.

75. A method according to claim 74, wherein said bag holder is immersed into said tank through an opening at said invariant immersion velocity.

76. A method according to any one of claims 73 to 75, comprising means to prevent ratio between that of the surface area S and that of the volume V of said biological material from increasing during the immersion to more than 4:3.

77. A method according to any one of claims 75 or 76, comprising engaging walls of said bag holder by a guide member extending from said opening into said tank and adapted for engaging walls of said bag holder, when the bag holder is being immersed into the tank via said opening, to reduce widening of the bag during its being frozen by the cryogenic fluid.

78. A method according to claim 77, wherein the guide member comprising a plurality of guides in the form of tongues depending into said tank from said opening.

79. A method according to Claim 78, wherein the guides are spaced by a proximal distance at a proximal portion of the guide member and a distal distance at a distal portion of the guide member, said distal distance being equal to or lesser than said proximal distance.

80. A method according to any one of claims 77 to 79, wherein said tank comprises a cover, wherein the proximal portion of the guide member is attached to said cover at the opening's periphery.

81. A method according to claim 80, wherein the cover has an inner surface facing the interior of the tank, and the proximal portion is attached to the inner surface.

82. A method according to any one of claims 74 to 81, comprising keeping level of said cryogenic fluid in said tank within no less than 3 cm below the maximal fluid level during the immersion of the bag with the biological material.

83. A method according to any one of claims 74 to 82, comprising monitoring a level of the cryogenic fluid within the tank by level sensors.

84. A method according to any one of claims 73 to 83, wherein the bag holder is in the form of a cartridge comprising a pair of frame members.

85. A method according to claim 84, wherein said frame members constitute side walls of the cartridge, for placing the bag with the biological material in a space created therebetween.

86. A method according to claim 85, wherein the bag is of a shape substantially complementary to a shape of said space.

87. A method according to any one of claims 84 to 86, wherein the frame members are perforated to reduce the thermal mass of the frame.

88. A method according to any one of claims 84 to 87, wherein the frame members comprise a common pivot axis.

89. A method according to claim 88, comprising moving the frame members with respect to said pivot axis and with respect to each other, thereby changing a position of the bag holder between an open and a closed positions.

90. A method according to any one of claims 89, wherein the bag holder, while in said closed position, has a shape that corresponds to a shape of the opening.

91. A method according to any one of claims 73 to 90, wherein the bag holder comprises stiffening members for keeping a width of the bag uniform.

92. A method according to any one of claims 73 to 91, comprising a temperature control block for setting an immersion temperature of said bag, prior to said immersion, to a pre-determined value higher than a temperature of said cryogenic fluid.

93. A method according to claim 92, wherein said temperature control block is adapted for setting said immersion temperature to between about 0°C and about 30°C.

94. A method according to any one of claims 73 to 93, wherein the cryogenic fluid is a liquid.

95. A method for cryopreserving a liquid biological material disposed in a bag having a longitudinal axis, the method comprising:

- placing the bag in a bag holder; and
- immersing said bag holder into a cryogenic fluid in a direction parallel to said longitudinal axis at an invariant immersion velocity selected so as to ensure that cooling behavior of the biological material at least at three different locations along the longitudinal axis of the bag is defined by a curve having initial curved portion, central essentially linear portion and final curved portion, the central portions corresponding to more than half of the range of

temperatures, and at least the central portions being parallel at said at least three locations.

96. A method according to claim 95, wherein said bag holder is immersed into a tank containing said cryogenic fluid.

97. A method according to claim 96, wherein said bag holder is immersed into said tank through an opening at said invariant immersion velocity.

98. A method according to any one of claims 95 to 97, comprising means to prevent ratio between that of the surface area S and that of the volume V of said biological material from increasing during the immersion to more than 4:3.

99. A method according to any one of claims 97 to 98, comprising engaging walls of said bag holder a guide member extending from said opening into said tank and adapted for engaging walls of said bag holder, when the bag holder is being immersed into the tank via said opening, to reduce widening of the bag during its being frozen by the cryogenic fluid.

100. A method according to claim 99, wherein the guide member comprising a plurality of guides in the form of tongues depending into said tank from said opening.

101. A method according to Claim 100, wherein the guides are spaced by a proximal distance at a proximal portion of the guide member and a distal distance at a distal portion of the guide member, said distal distance being equal to or lesser than said proximal distance.

102. A method according to any one of claims 99 to 101, wherein said tank comprises a cover, wherein the proximal portion of the guide member is attached to said cover at the opening's periphery.

103. A method according to claim 102, wherein the cover has an inner surface facing the interior of the tank, and the proximal portion is attached to the inner surface.

104. A method according to any one of claims 96 to 103, comprising keeping level of said cryogenic fluid in said tank within no less than 3 cm below the maximal fluid level during the immersion of the bag with the biological material.

105. A method according to any one of claims 96 to 104, comprising monitoring a level of the cryogenic fluid within the tank by level sensors.

106. A method according to any one of claims 95 to 105, wherein the bag holder is in the form of a cartridge comprising a pair of frame members.

107. A method according to claim 106, wherein said frame members constitute side walls of the cartridge, for placing the bag with the biological material in a space created therebetween.

108. A method according to claim 107, wherein the bag is of a shape substantially complementary to a shape of said space.

109. A method according to any one of claims 106 to 108, wherein the frame members are perforated to reduce the thermal mass of the frame.

110. A method according to any one of claims 106 to 108, wherein the frame members comprise a common pivot axis.

111. A method according to claim 110, comprising moving the frame members with respect to said pivot axis and with respect to each other, thereby changing a position of the bag holder between an open and a closed positions.

112. A method according to claim 111, wherein the bag holder, while in said closed position, has a shape that corresponds to a shape of the opening.

113. A method according to any one of claims 95 to 112, wherein the bag holder comprises stiffening members for keeping a width of the bag uniform.

114. A method according to any one of claims 95 to 113, comprising a temperature control block for setting an immersion temperature of said bag, prior to said immersion, to a pre-determined value higher than a temperature of said cryogenic fluid.

115. A method according to claim 114, wherein said temperature control block is adapted for setting said immersion temperature to between about 0°C and about 30°C.

116. A method according to any one of claims 95 to 115, wherein the cryogenic fluid is a liquid.

117. A system for warming a cryopreserved liquid biological material disposed in a bag, the system comprising:

- a heat source;
- a warming device having a space for placing said bag therein, connected to the heat source and adapted to transfer heat from the heat source to the bag;
- means for maintaining the heat source in heat transfer contact with a cryogenically preserved portion of said material to allow receiving said heat by said cryogenically preserved portion.

118. A system according to claim 117, wherein the warming device comprises an empty space for accommodating of any liquefied material apart from the cryopreserved material.

119. A system according to claim 118, wherein said space is a part of the bag.

120. A system according to any one of claims 117 to 119, wherein the warming device comprises a pair of plates creating said space for the bag therebetween.

121. A system according to claim 120, wherein at least one of said plates is used as the heat source.

122. A system according to any one of claims 120 or 121, wherein at least one of said plates is adapted to move with respect to the other plate in a direction perpendicular to a longitudinal axis of said bag, thereby applying pressure on the bag.

123. A system according to any one of claims 117 to 122, wherein the heat source is adapted to continually supply heat to the warming device until all the cryopreserved material is liquefied and removed.

124. A system according to any one of claims 117 to 123, wherein the warming device is adapted to store heat supplied by the heat source.

125. A system according to any one of claims 117 to 124, wherein the warming device comprising heat storage means and the heat source is adapted to supply heat to said heat storage means of the warming device only prior to a warming process, during which the heat stored in the warming device is supplied to the bag.

126. A system according to any one of claims 117 to 125, wherein said bag has a cryopreserved material section filled with the cryopreserved material before said warming and an empty section constituting said empty space, which is free of the cryopreserved material and is adapted to accommodate said liquefied material.

127. A system according to any one of claims 117 to 126, wherein the heat source is an electrical heater.

128. A method for warming a cryopreserved liquid biological material disposed in a bag, the method comprising:

- providing a heat source;
- providing a warming device and placing said bag within a space therein;
- connecting the warming device to the heat source adapted to transfer heat from the heat source to the bag; and

- removing of a liquefied material thereby preventing it from receiving said heat.

129. A method according to claim 128, comprising accommodating of said liquefied material apart from the cryopreserved material in an empty space of said bag.

130. A method according to claim 129, wherein the warming device comprises a pair of plates creating said space for the bag therebetween.

131. A method according to claim 130, using at least one of said plates as heat source.

132. A method according to any one of claims 130 or 131, comprising moving of at least one of said plates with respect to the other plate in a direction perpendicular to a longitudinal axis of said bag, thereby applying pressure on the bag.

133. A method according to any one of claims 128 or 132, comprising continually supplying heat to the warming device by the heat source until all the cryopreserved material is liquefied and removed.

134. A method according to any one of claims 128 or 133, wherein the warming device is adapted to store heat supplied by the heat source.

135. A method according to any one of claims 128 or 134, wherein the warming device comprising heat storage means and the heat source is adapted to supply heat to said heat storage means of the warming device only prior to a warming process, during which the heat stored in the warming device is supplied to the bag.

136. A method according to any one of claims 128 or 135, wherein said bag has a cryopreserved material section filled with the cryopreserved material before said warming and an empty section constituting said empty space, which is free of the cryopreserved material and is adapted to accommodated said liquefied material.

137. A method according to any one of claims 128 or 136, wherein the heat source is an electrical heater.

138. A method according to any one of claims 128 or 137, wherein the heat source is a warm water.

139. A system for warming a cryopreserved liquid biological material disposed in a bag having a longitudinal axis, the system comprising:

- a heat source;

- a warming device having a space for placing said bag therein and fluid accommodating means for accommodating a fluid therein, the device adapted to transfer heat from the fluid to the bag; and
- means for maintaining a liquefied material within the bag evenly dispersed along said longitudinal axis thereby improving heat transfer between said fluid and said cryopreserved material.

140. A system according to claim 139, wherein the warming device comprises a pair of plates creating said space for the bag therebetween.

141. A system according to claim 140, wherein the fluid accommodating means are channels comprised within at least one of said plates.

142. A system according to any one of claims 139 to 141, wherein the device comprises an empty space for accommodating of any liquefied material apart from the cryopreserved material.

143. A system according to any one of claims 140 to 142, wherein at least one of said plates is adapted to move with respect to the other plate in a direction perpendicular to said axis, thereby applying pressure on the bag.

144. A system according to any one of claims 139 to 143, comprising an immersion tank filled with said fluid.

145. A system according to claim 144, comprising a pump for pushing said fluid from said tank to said fluid accommodating means.

146. A system according to any one of claims 139 to 145, wherein the fluid is warm water.

147. A method for warming a cryopreserved liquid biological material disposed in a bag having a longitudinal axis, the method comprising

- providing a heat source;
- providing a warming device having a space for placing said bag therein and fluid accommodating means for accommodating a fluid therein, for transferring heat from the fluid to the bag;
- providing for maintaining a liquefied material within the bag evenly dispersed along said longitudinal axis thereby improving heat transfer between said fluid and said cryopreserved material; and
- removing from the bag a liquefied material thereby preventing it from receiving said heat during warming of cryopreserved material left in the bag.

148. A method according to claim 147, wherein the warming device comprises a pair of plates creating said space for the bag therebetween.

149. A method according to claim 148, wherein the fluid accommodating means are channels comprised within at least one of said plates.

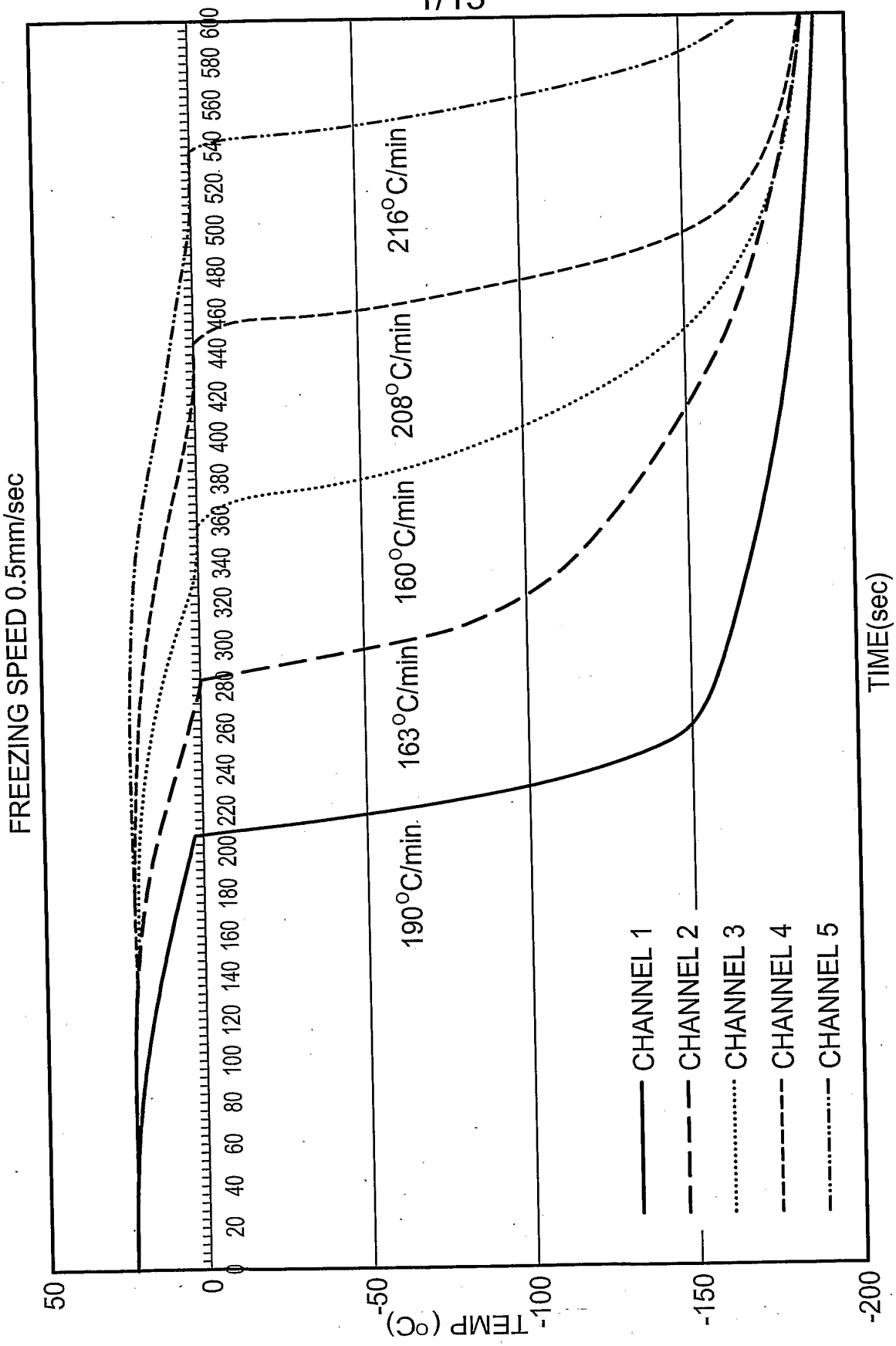
150. A method according to any one of claims 147 to 149, comprising removing from the bag a liquefied material thereby preventing it from receiving said heat during warming of cryopreserved material left in the bag.

151. A method according to any one of claims 148 to 149, comprising moving at least one of said plates with respect to the other plate in a direction perpendicular to said axis, thereby applying pressure on the bag.

152. A method according to any one of claims 148 to 151, comprising providing an immersion tank filled with said fluid.

153. A method according to claim 152, comprising providing a pump for pushing said fluid from said tank to said fluid accommodating means.

154. A method according to any one of claims 148 to 153, wherein the fluid is warm water.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TIME(sec)
TEMP (°C)
50
0
-50
-100
-150
-200
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440 460 480 500 520 540 560 580 600
190°C/min
163°C/min
160°C/min
208°C/min
216°C/min
CHANNEL 1
CHANNEL 2
CHANNEL 3
CHANNEL 4
CHANNEL 5

Fig. 1A

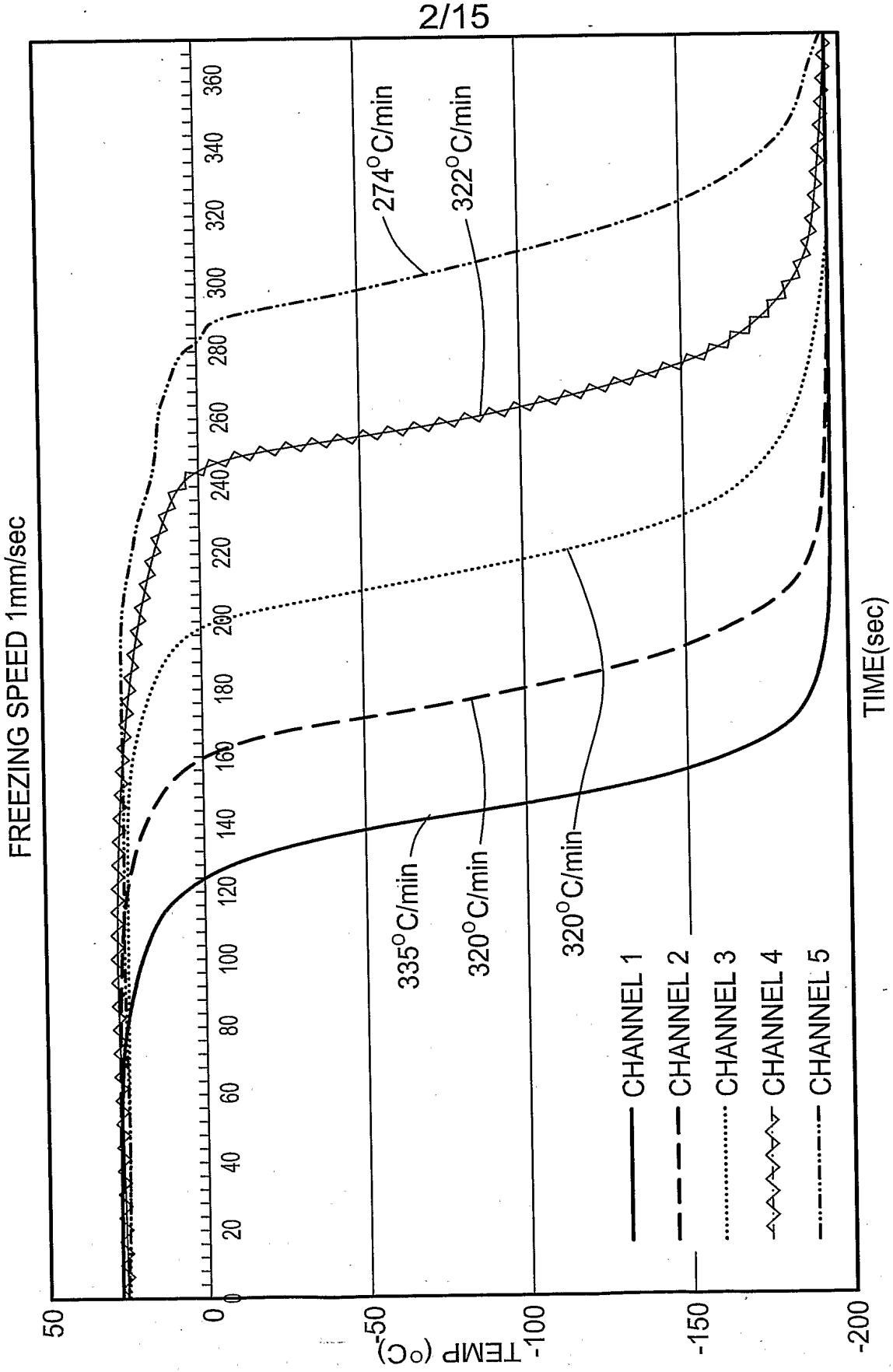
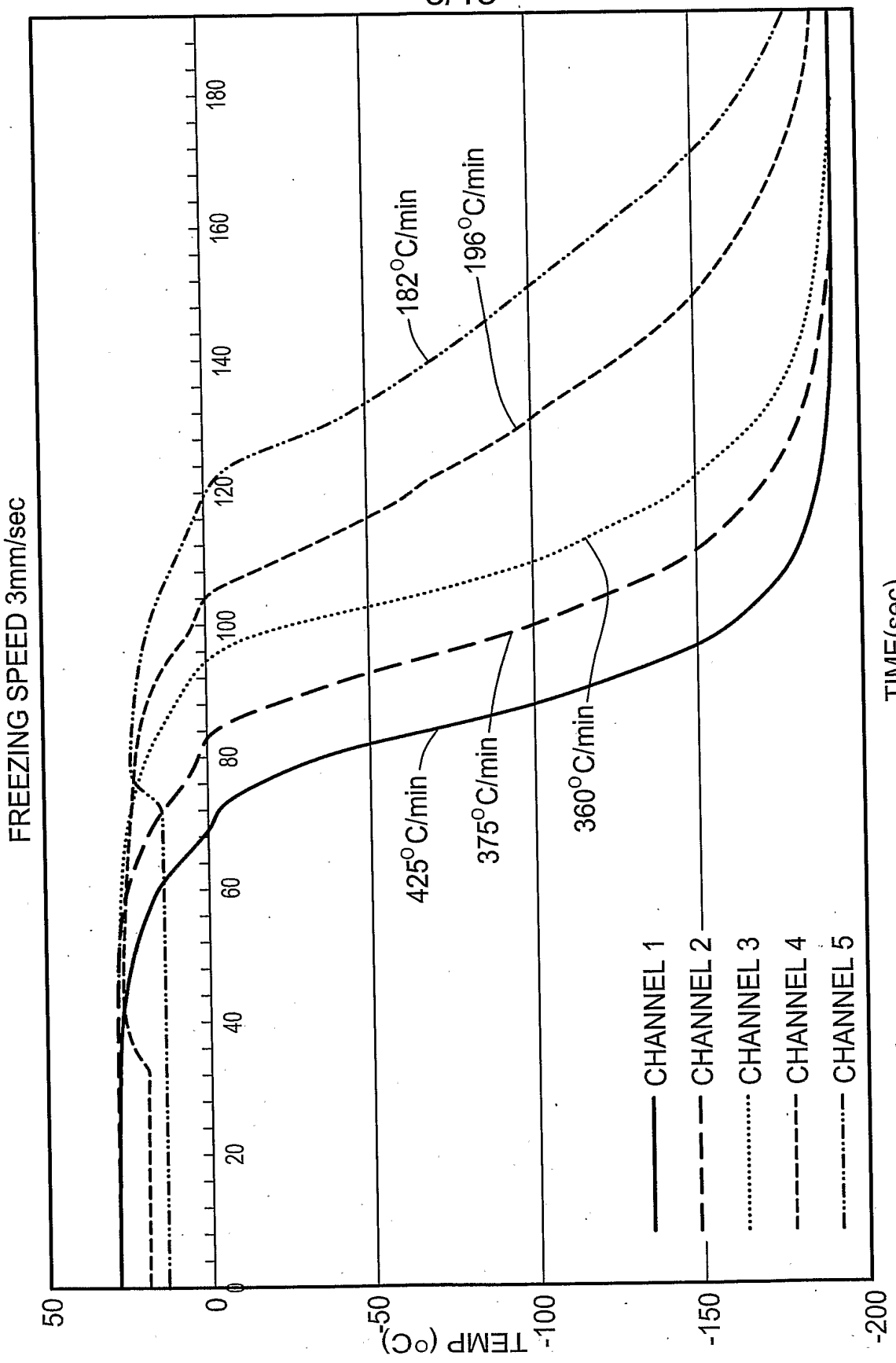

FREEZING SPEED 1mm/sec
TIME(sec)
TEMP (°C)
50
0
-50
-100
-150
-200
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
274°C/min
322°C/min
335°C/min
320°C/min
320°C/min
CHANNEL 1
CHANNEL 2
CHANNEL 3
CHANNEL 4
CHANNEL 5

Fig. 1B


FREEZING SPEED 3mm/sec
TIME(sec)
TEMP (°C)
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0
-50
-100
-150
-200
0
20
40
60
80
100
120
140
160
180
182°C/min
196°C/min
425°C/min
375°C/min
360°C/min
CHANNEL 1
CHANNEL 2
CHANNEL 3
CHANNEL 4
CHANNEL 5

Fig. 1C

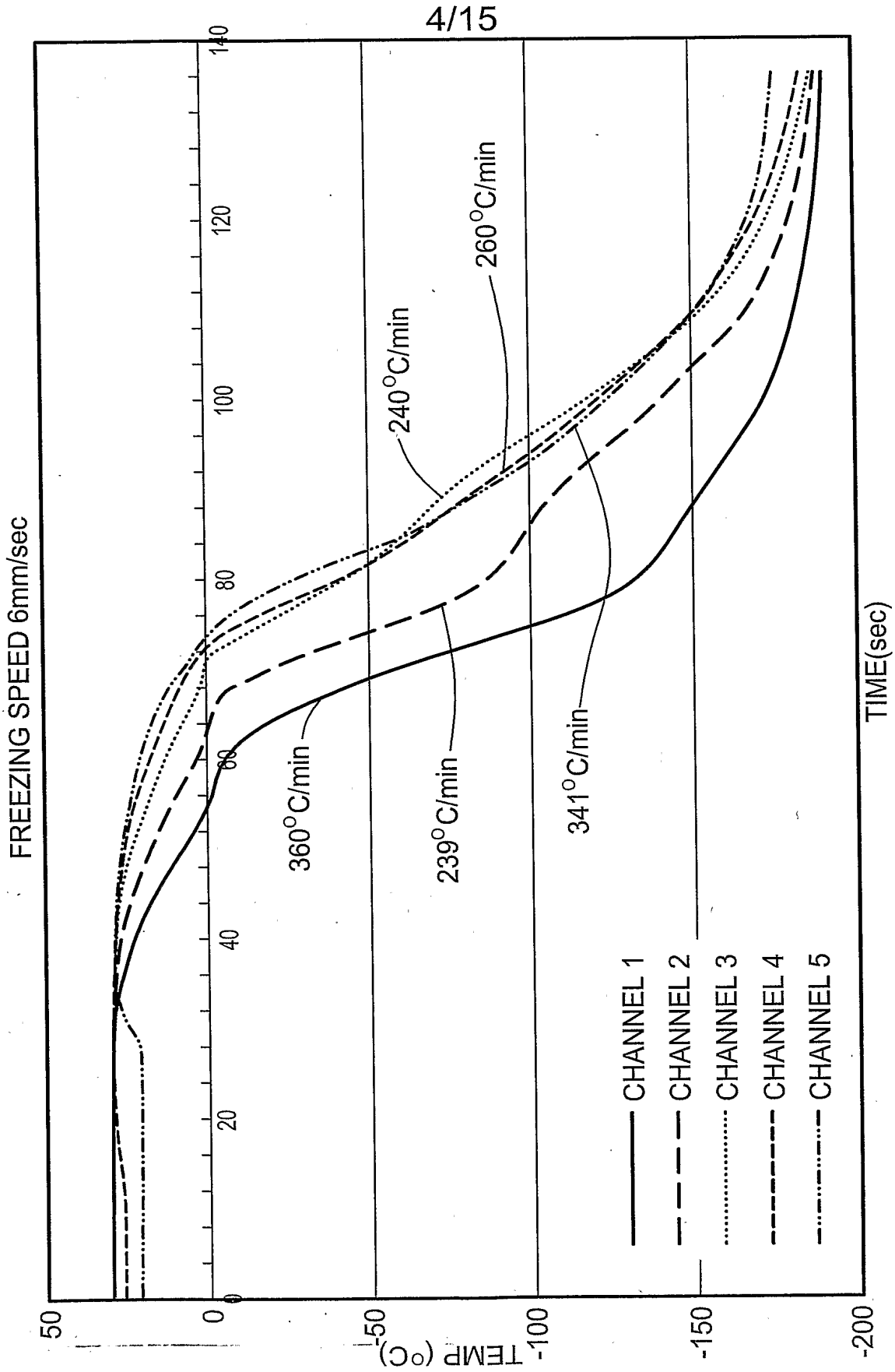

FREEZING SPEED 6mm/sec
TIME(sec)
TEMP (°C)
50
0
-50
-100
-150
-200
0
20
40
60
80
100
120
140
360°C/min
239°C/min
341°C/min
240°C/min
260°C/min
CHANNEL 1
CHANNEL 2
CHANNEL 3
CHANNEL 4
CHANNEL 5

Fig. 1D

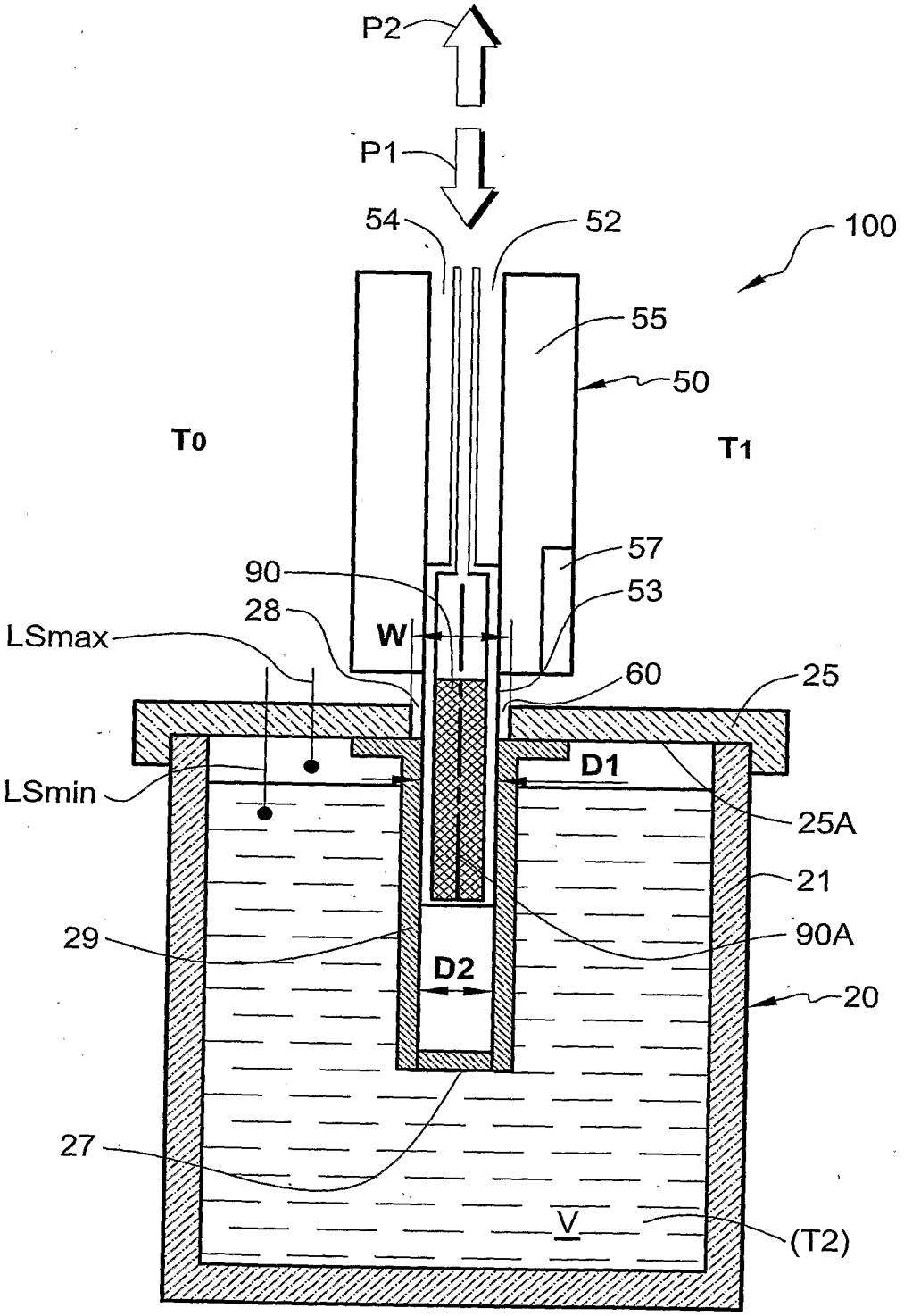

P2
P1
54
52
100
55
50
T0
T1
57
90
53
28
W
LSmax
60
25
D1
LSmin
25A
21
29
90A
D2
20
27
V
(T2)

Fig. 2

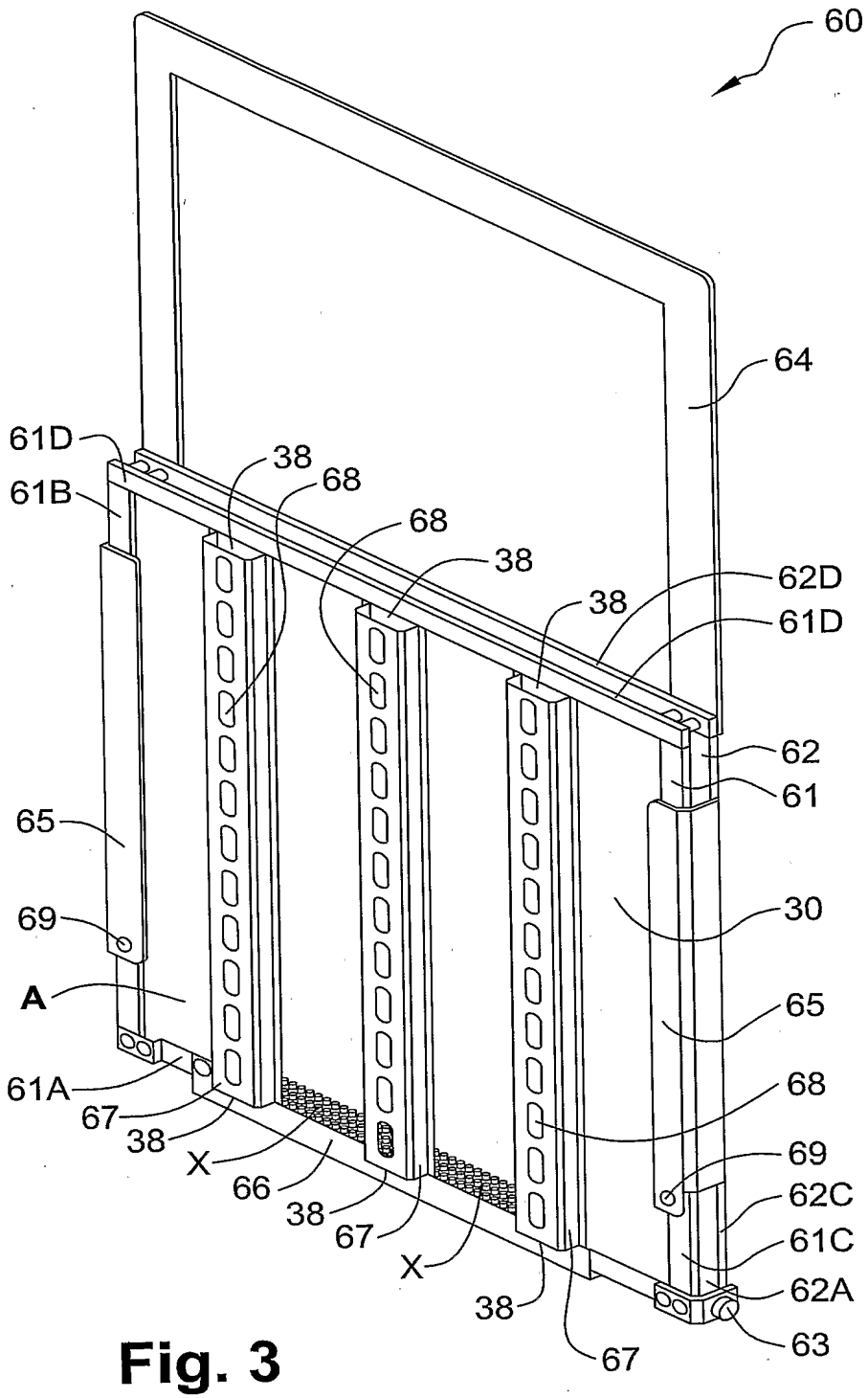

60
64
61D
61B
38
68
68
38
38
62D
61D
62
61
65
69
30
A
65
61A
67
38
X
66
38
67
X
38
67
68
69
62C
61C
62A
63

Fig. 3

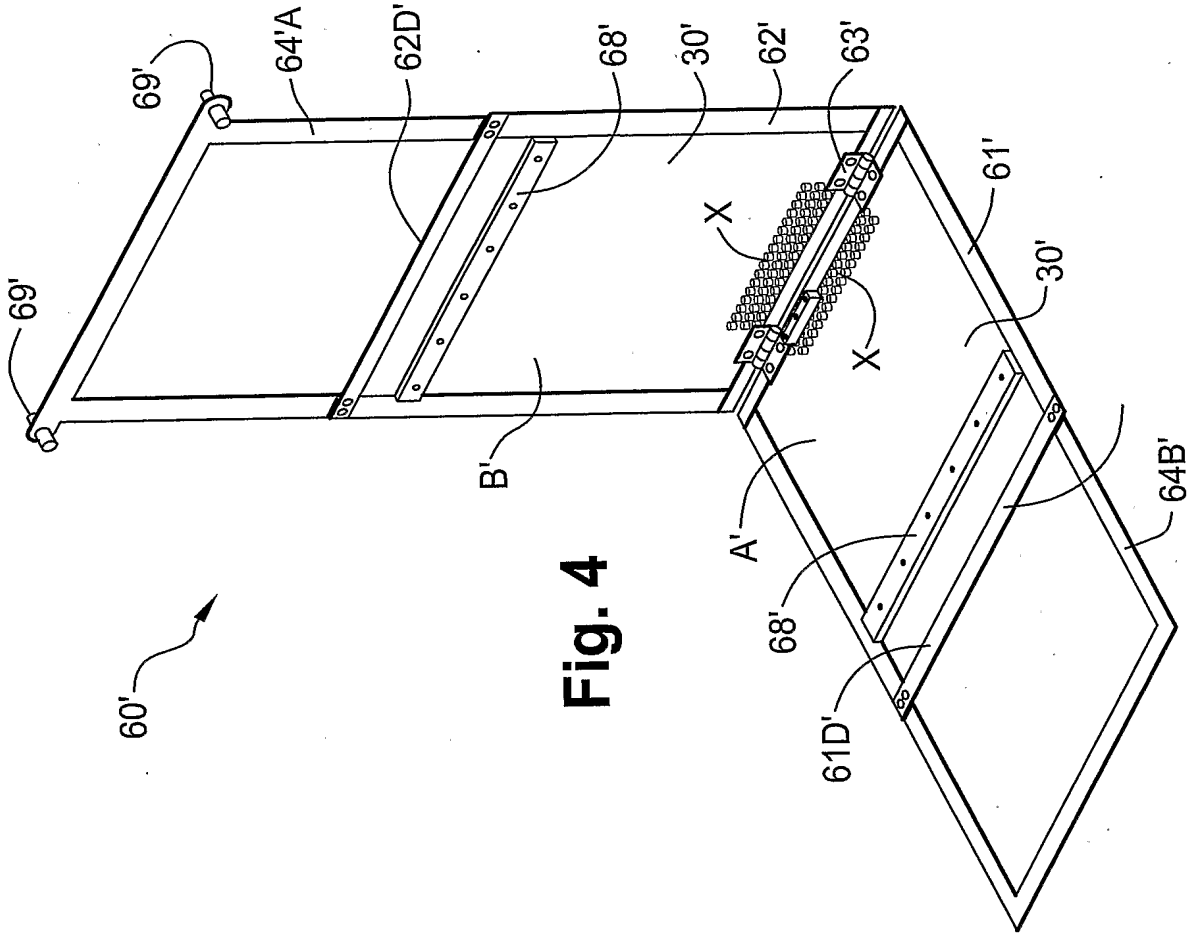

69'
64'A
62D'
68'
30'
62'
63'
61'
X
X
30'
69'
B'
A'
64B'
68'
61D'
60'

Fig. 4

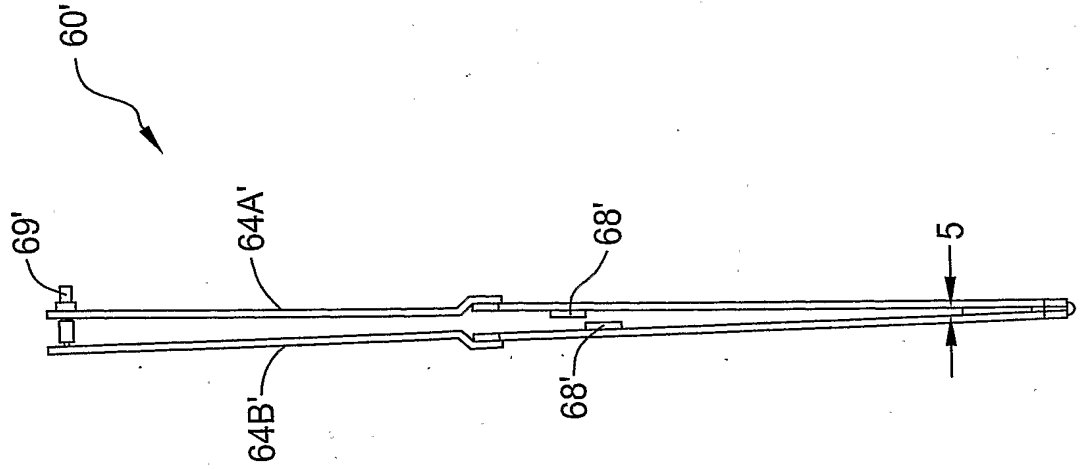

60'
69'
64A'
68'
5
64B'
68'

Fig. 4A

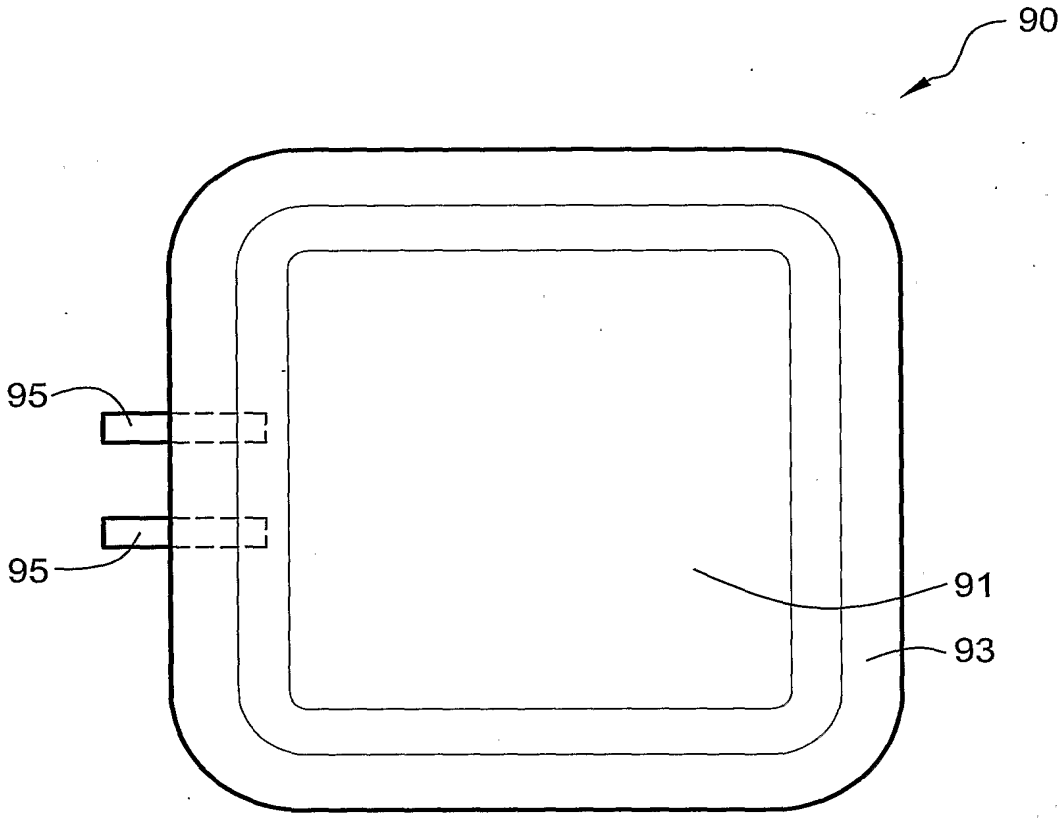


Fig. 5A

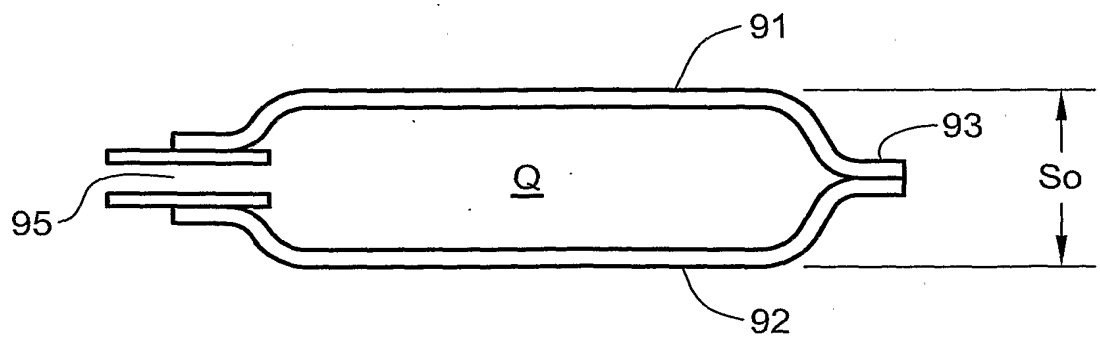


Fig. 5B

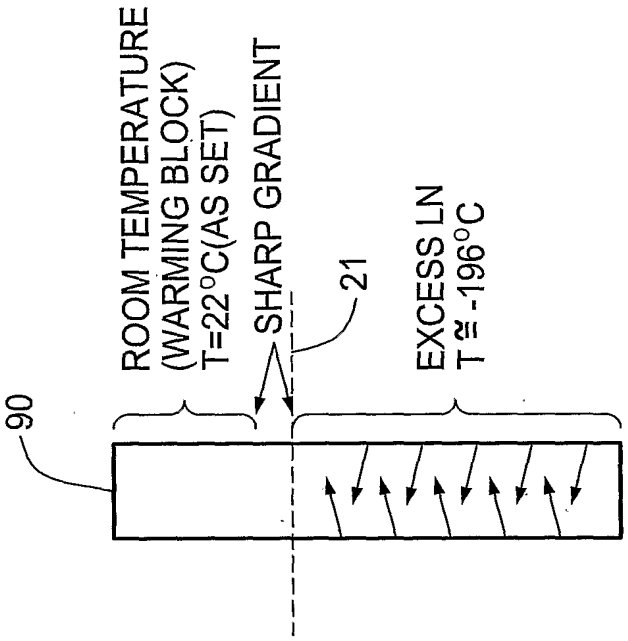

90
ROOM TEMPERATURE
(WARMING BLOCK)
T=22°C(AS SET)
SHARP GRADIENT
21
EXCESS LN
T ≅ -196°C

Fig. 6A

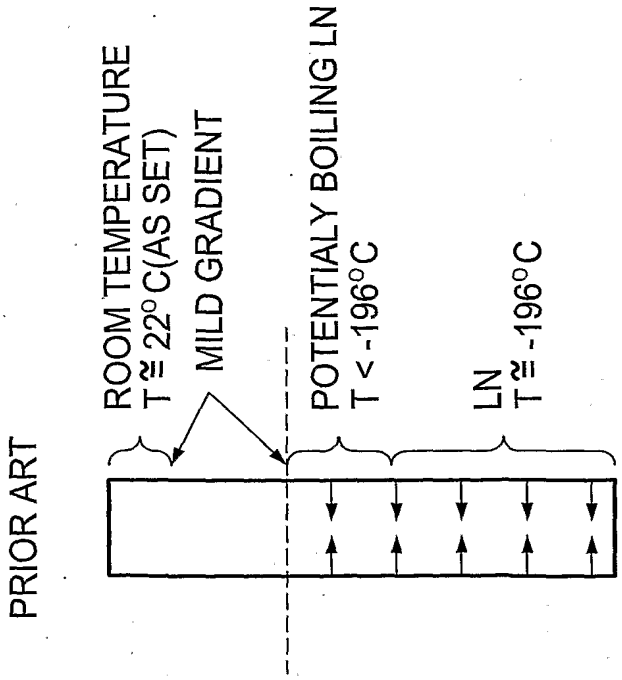

PRIOR ART
ROOM TEMPERATURE
T ≅ 22°C(AS SET)
MILD GRADIENT
POTENTIALY BOILING LN
T < -196°C
LN
T ≅ -196°C

Fig. 6B

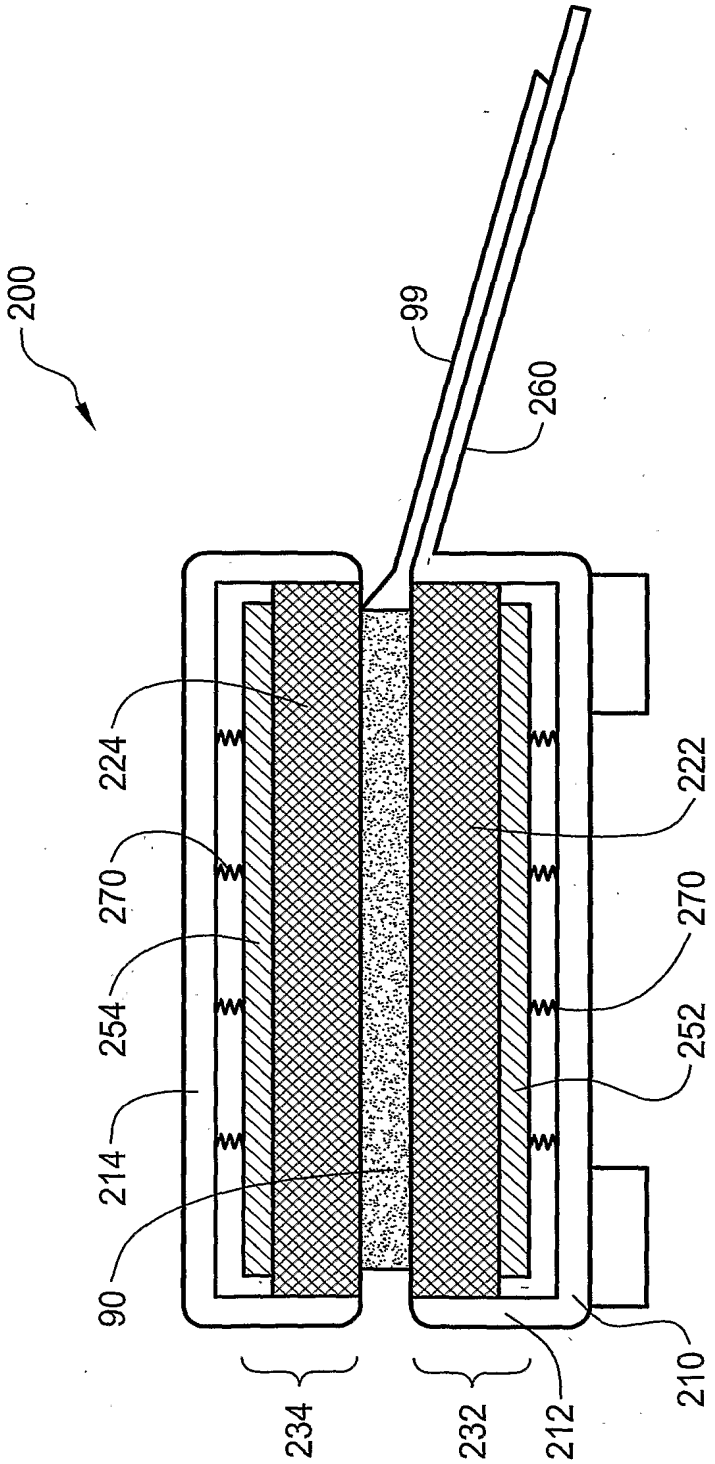


Fig. 7

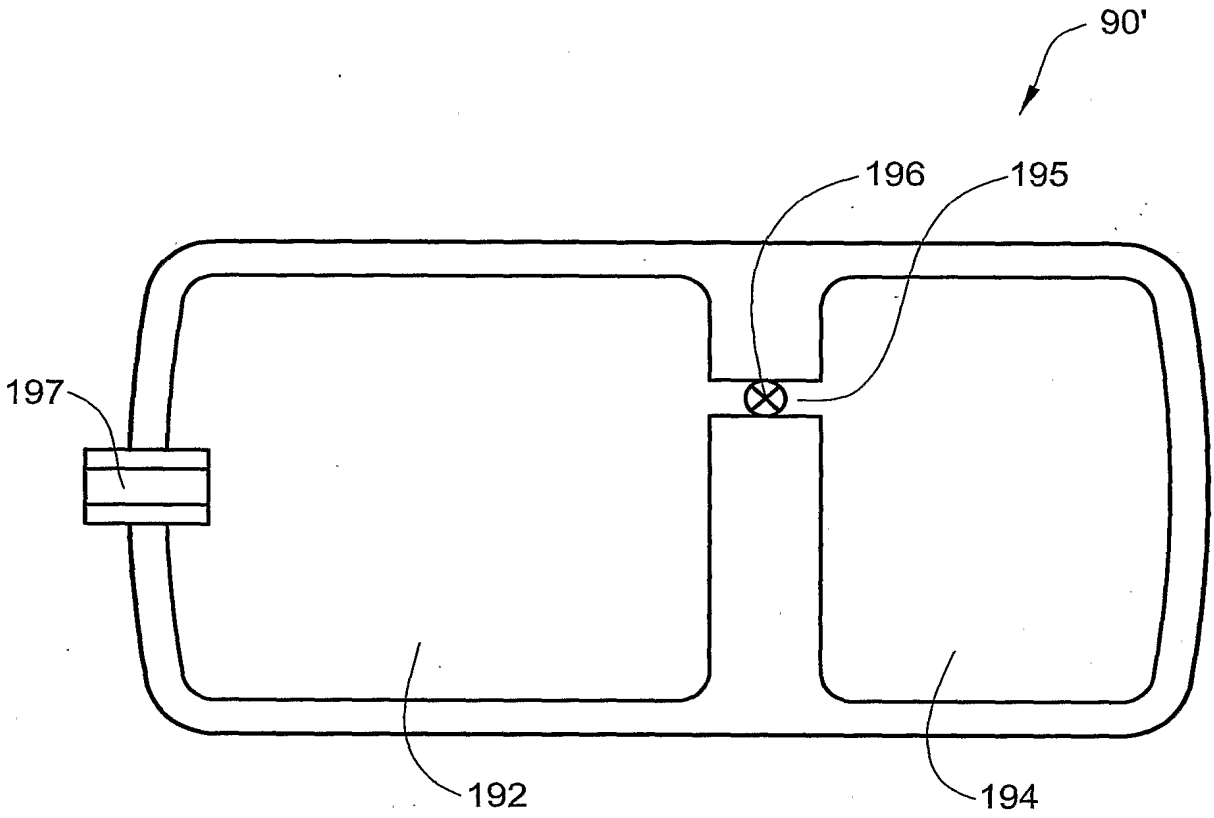


Fig. 8

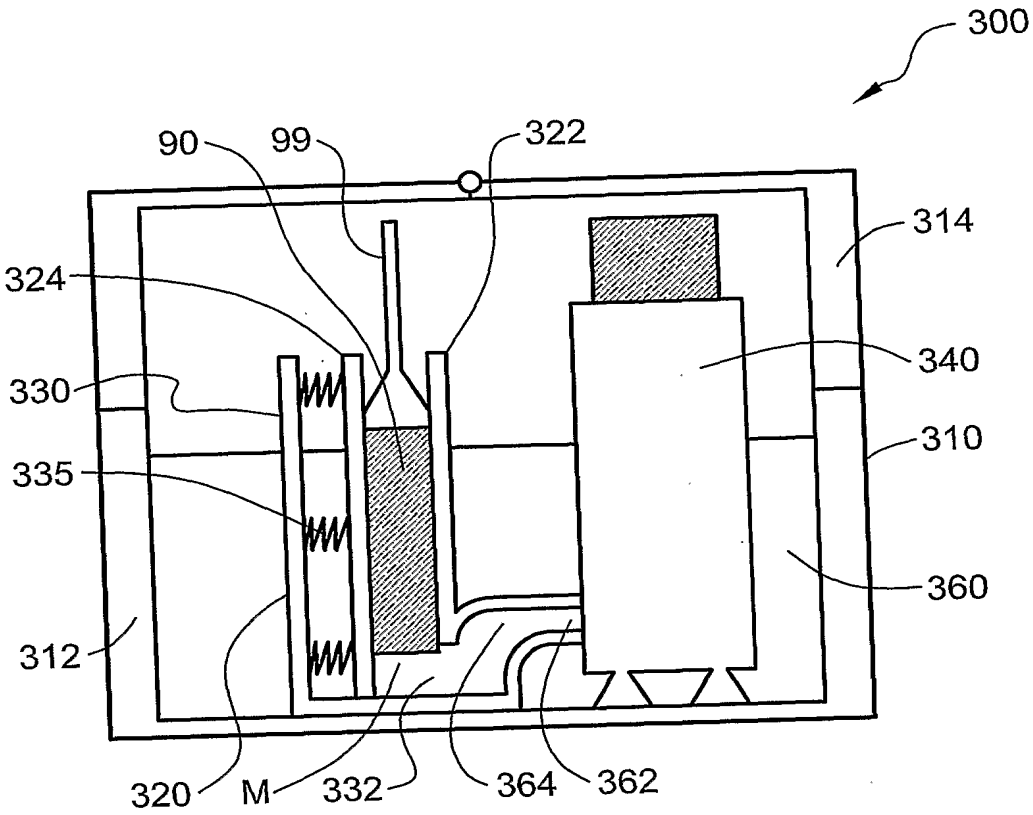

300
90
99
322
314
324
340
330
310
335
360
312
320
M
332
364
362

Fig. 9A

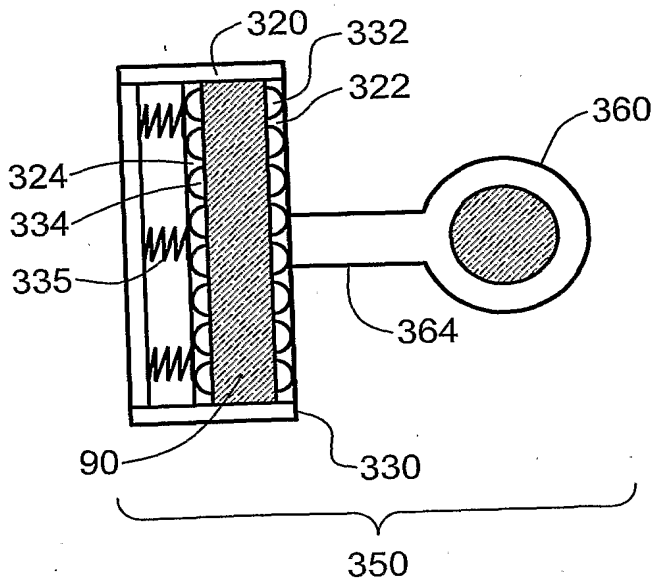

320
332
322
360
324
334
335
364
90
330
350

Fig. 9B

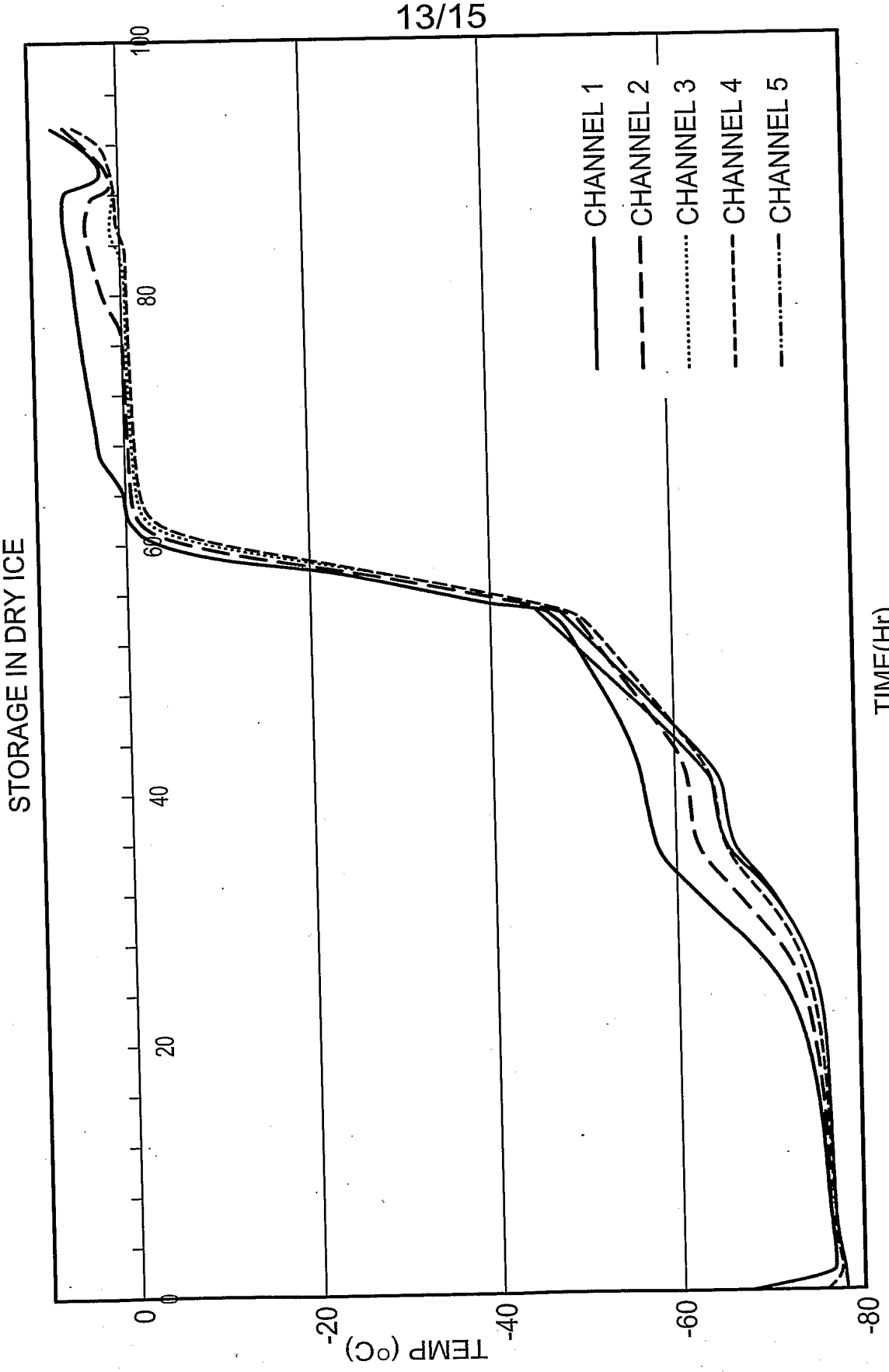
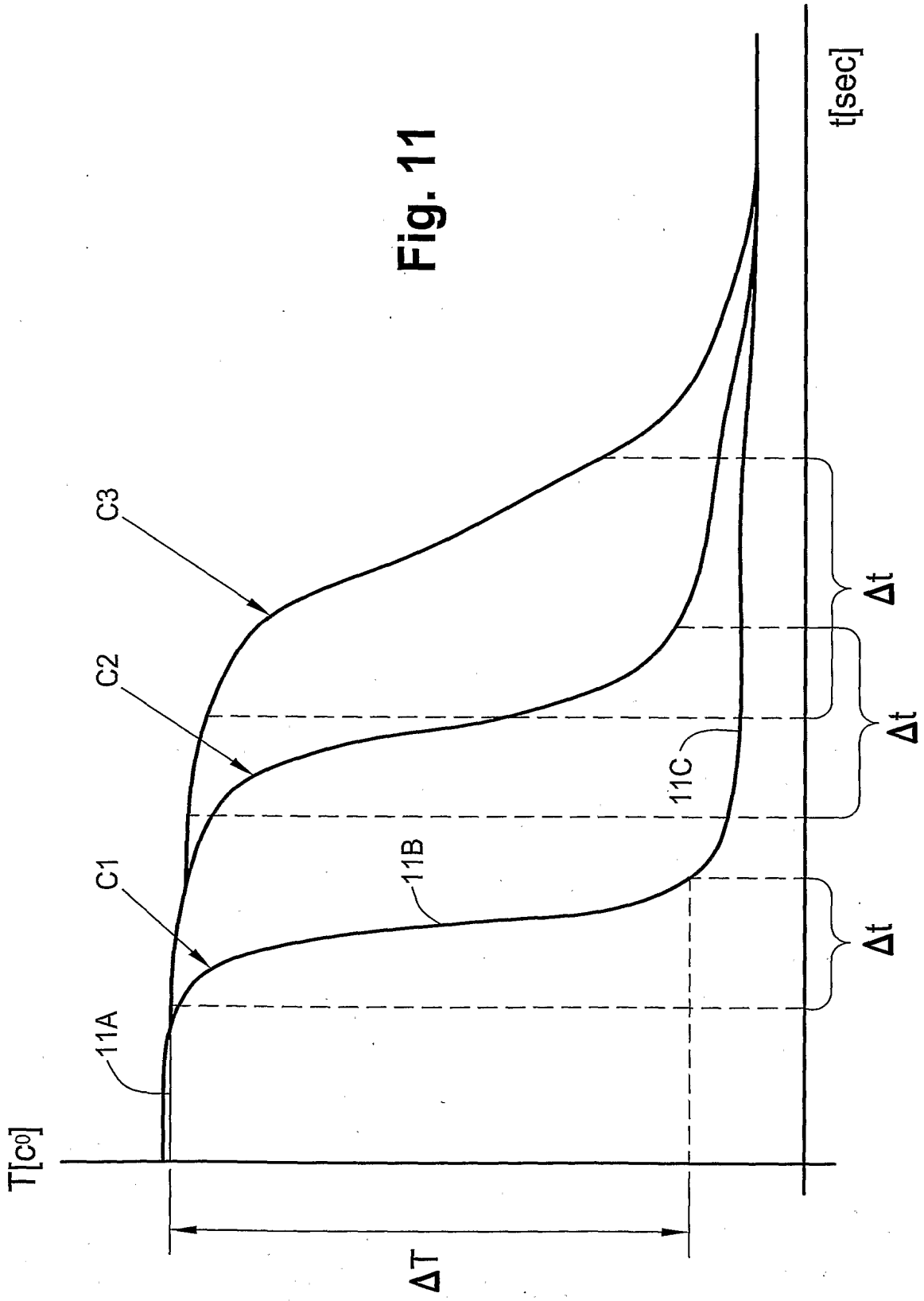

STORAGE IN DRY ICE
TIME(Hr)
TEMP (°C)
0
20
40
60
80
100
0
-20
-40
-60
-80
CHANNEL 1
CHANNEL 2
CHANNEL 3
CHANNEL 4
CHANNEL 5

Fig. 10

Fig. 11



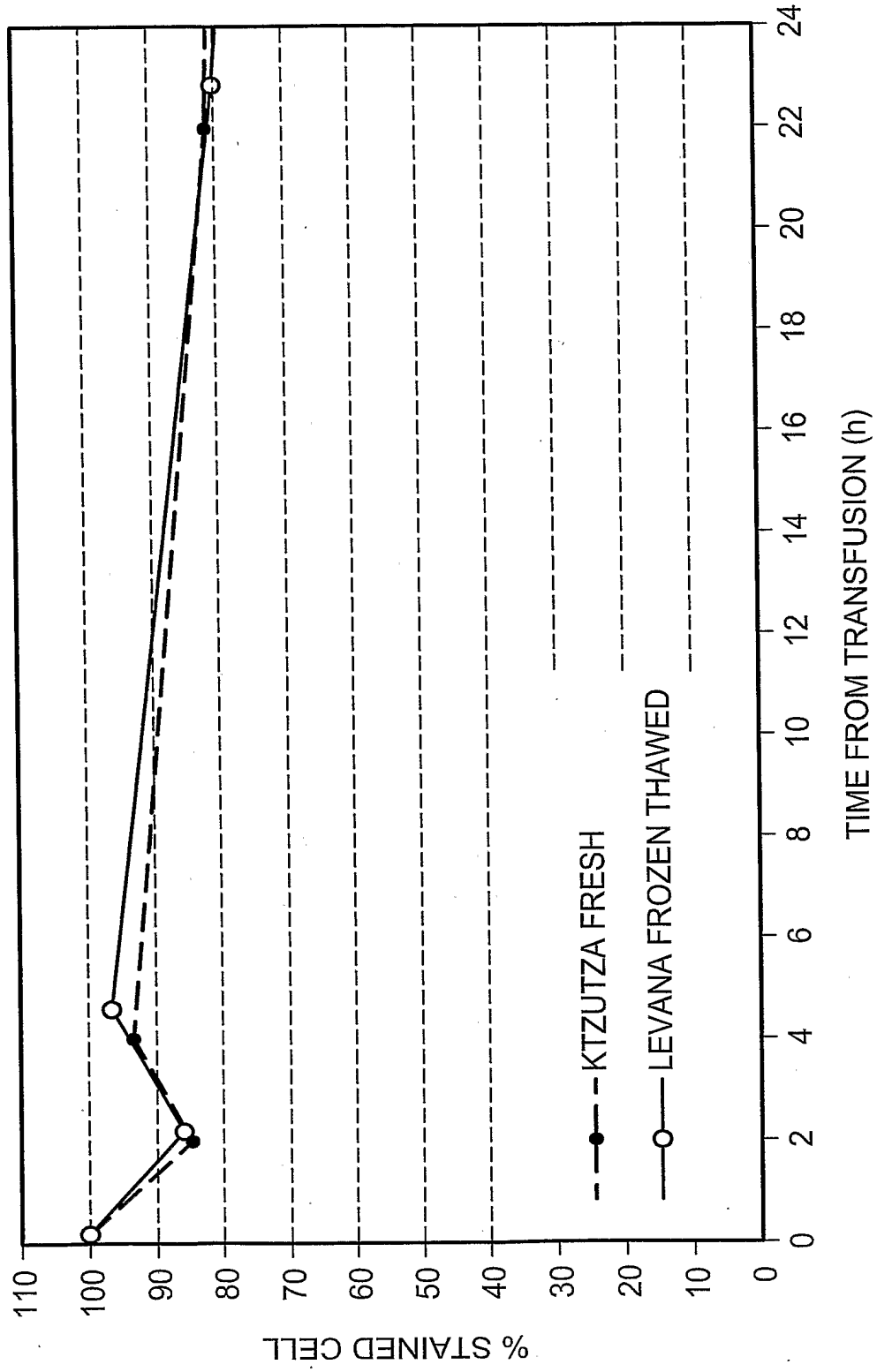

% STAINED CELL
110
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME FROM TRANSFUSION (h)
KTZUTZA FRESH
LEVANA FROZEN THAWED

Fig. 12